Journal of Virological Methods 265 (2019) 35–41

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Recombinant DENV 2 NS5: An effective antigen for diagnosis of DENV infection

Ting Zhang[a], Ming-Lian Wang[a,*], Gui-Rong Zhang[b], Wei Liu[a], Xiang-Qian Xiao[a], Yi-Shu Yang[a], Jin-Tao Li[a], Zhi-Ming Xun[a,c], Dan-Yang Li[a], Paul K.S. Chan[d]

[a] College of Life Science and Bioengineering, Beijing University of Technology, Beijing, China
[b] Peking Medriv Academy of Genetics and Reproduction, Beijing, China
[c] Beijing Chaoyang Hospital, Beijing, China
[d] Department of Microbiology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong, China

ARTICLE INFO

Keywords:
Dengue virus
Nonstructural protein 5
DENV infection diagnosis

ABSTRACT

Dengue fever is a mosquito-borne viral disease with dramatically increasing morbidity rate worldwide in decades. Since there is no specific treatment to date, early diagnosis is important for providing proper timely medical care to minimize mortality, and for the prompt initiation of public health control measures. NS5 is a potential biomarker for dengue virus infection due to its highly conserved and immunogenic properties. In this study, the DENV 2 NS5 full-length and the DENV 2 NS5 C-terminus RNA-dependent RNA polymerase domain fragment (NS5-C70) expression plasmids were constructed, and the 104 kDa full-length NS5 and the 70 kDa NS5-C70 were respectively expressed in *Escherichia coli*. These two purified recombinant products were found to react with the sera of patients infected with dengue virus when analyzed by an enzyme-linked immunosorbent assay (ELISA), which resulted in significantly higher absorption values than those of control sera. The recombinant DENV 2 NS5 exhibited strong reactivity to each of the four types of sera, whereas the NS5-C70 showed strong reactivity only to DENV 2 and 4. In comparison, the positive agreement value of recombinant NS5-based assay with either MyBioSource or Panbio assay was higher than that of the two commercially available IgG indirect ELISA kits. These results suggest that the recombinant DENV 2 NS5 be an effective antigen for detection of dengue virus infection. The recombinant NS5-C70 may also be used as an auxiliary antigen for diagnostic purposes.

1. Introduction

1.1. Dengue fever and dengue virus

Dengue fever (DF) is one of the most common arthropod borne viral diseases in humans, endemically in tropical and subtropical regions, designated as a major international public health concern by the World Health Organization (WHO). There are nearly 390 million dengue virus (DENV) infections annually, 20–25% with clinically apparent symptoms (Ayukekbong et al., 2017). For the past decades, DF has spread from less than 9 to currently about 128 countries (Brady et al., 2012; Ebi and Nealon, 2016). DENV belongs to the *Flaviviridae* family, and is generally classified into four immunologically distinct serotypes, DENV 1 to 4. All four serotypes can be transmitted from human to human by mosquitoes, mainly *Aedes aegypti* and *Aedes albopictus* (Rosen and Gubler, 1974), and cause diseases ranging from non-specific febrile illness to classic DF, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), with the latter two manifestations leading to possible mortality (Bhatt et al., 2013). DENV 2 cause highest infection and mortality rates among all the four serotypes (Balasubramanian et al., 2017) Individuals infected by any one serotype will have life-long immunity against reinfection of the same serotype, but only partially protected from other serotypes. Infections with a different serotype are associated with a higher risk of severe complications as DHF and/or DSS due to antibody-dependent enhancement (ADE) (Stephenson, 2005).

DENV is a single-stranded positive sense RNA virus with its 96% genome forming a large open reading framework (ORF) (Kuhn et al., 2002), encoding a polyprotein of about 3400 amino acids with a molecular weight of about 380 kDa (Halstead, 2007; Vicente et al., 2016). The polyprotein is later processed by viral and host proteases generating ten mature viral proteins: three structural proteins (capsid [C],

* Corresponding author.
E-mail address: mlw@bjut.edu.cn (M.-L. Wang).

https://doi.org/10.1016/j.jviromet.2018.12.005
Received 1 February 2018; Received in revised form 13 December 2018; Accepted 15 December 2018
Available online 16 December 2018

membrane [M], and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Mackenzie, 2010; Paul and Bartenschlager, 2015). DENV is also a prototype of other flaviviruses, for example the West Nile virus (WNV) and the Zika virus (Sahili and Lescar, 2017). Their homologies raise the risk of cross-reactive immune responses and subsequent occurrence of ADE in cases of secondary infections or in vaccinated individuals (Singh et al., 2017).

Some dengue endemic countries attempted to eradicate *Aedes aegypti,* but have not gained success (Shu and Huang, 2004). The effective control of DF would require an efficacious vaccine and antiviral therapies to all serotypes, strong public health education and control measures supported by diagnostic methods with comprehensive validations (Scott, 2016).

Around early 2016, the first dengue vaccine, Dengvaxia (CYD-TDV), was marketed in several countries (Halstead and Russell, 2016). However, accumulating reports suggest that this vaccine has not achieved the desired effect. The reports indicate increased risk of hospitalization and severe cases among young children during the follow-up in years 3–6 (Halstead and Russell, 2016; Sabchareon, 2015; Sophia et al., 2016; Wichmann et al., 2017). The report of the emergence of a new viral type, DENV 5, in Malaysia may increase ADE and present additional challenge for vaccine development (Normile, 2013). To date, there is no effective anti-DENV drugs, but only symptomatic treatments. Early virus identification and timely treatment are the keys to reducing this disease and associated mortality.

1.2. Dengue diagnosis

The laboratory diagnostic methods for DENV infection include: i) Virus culture. Sera or plasma from patients with suspected dengue are inoculated into mosquito or mammalian cell lines (Shu and Huang, 2004). After virus isolation, serotype is identified by immunofluorescence using serotype-specific monoclonal antibodies (Bäck and Lundkvist, 2013). Although this method is considered as the gold standard of laboratory diagnosis of acute DENV infection (Kumarasamy et al., 2007), at least 6–10 days are required (Shu and Huang, 2004), and routine use is limited in endemic countries by the need for BSL-2 (or higher) laboratories and appropriately trained personnel. ii) Viral RNA detection. DENV RNA can be detected by polymerase chain reaction (PCR) from sera of patients during the acute phase (Johnson et al., 2005). Common genomic regions for PCR include E, NS1, E/NS1, prM/E. Viral load can be quantified by RT-PCR and strain typing can be carried out by nucleotide sequencing and phylogenetic analysis (Chow et al., 1998). However, this method is only applicable in the short viraemic phase ($\leq$ 5 days after onset of symptoms) (Wu et al., 2001). PCR also requires molecular laboratory setup which may not be feasible in some remote endemic area. iii) Antigen detection. NS1 is the main antigen target. The NS1 antigen can be detected by enzyme-linked immunosorbent assay (ELISA) in primary and secondary infected patients up to 9 days after onset of illness (Dussart et al., 2006; Peeling et al., 2010). iv) IgG or IgM antibody detection. NS1 is also the main viral antigen used to detect IgG or IgM antibodies in human sera or plasma (Blacksell et al., 2011). Some diagnostic tests by NS1 antigen or NS1-specific antibody detection are now commercially available (Pal et al., 2014), but most NS1-based assays have limited sensitivity, especially to DENV 4 serotype (Gelanew and Hunsperger, 2018; Hermann et al., 2014), and serological cross-reactivity among the flavivirus groups is common at the level of IgG (Makino et al., 1994). It is reported that the WNV NS5-based assay reliably discriminates between WNV infections and DENV or St. Louis encephalitis virus infections (Wong et al., 2003). NS1-specific antibody test requires the use of NS1 from all four serotypes. Although DENV 5 is assumed of a very low transmission rate, future new outbreaks cannot be ruled out (Mustafa et al., 2015). There are no reports on diagnosis of DENV 5 infection yet, which poses a challenge to the diagnosis of DENV infection (Taylor-Robinson, 2016).

1.3. DENV NS5 and NS5 RdRp domain

DENV NS5 are highly conserved among all of the *Flavivirus* that have not been found to participate in the ADE phenomenon (Beltramello et al., 2010; Dejnirattisai et al., 2010). NS5 is the largest protein of DENV with a molecular weight of about 104 kDa containing 900 amino acids, and is the most conserved protein encoded by the DENV genome, and its conservation is also reflected in the conformational architecture (Sahili and Lescar, 2017). The DENV NS5 is highly immunogenic (Lim et al., 2015), consisting of two major functional/structural domains, the N-terminal RNA methyltransferase (MTase) domain, residues 1–263, 30 kDa (Koonin, 1993), and the C-terminal RNA-dependent RNA polymerase (RdRp) domain, residues 272–900, 70 kDa (Koonin, 1991). The two domains are connected via a flexible linker (Lu and Gong, 2013).

DENV NS5 RdRp domain plays a vital role in viral RNA replication. Following viral entry and protein translation from the viral genome, NS5 RdRp domain is subjected to *de novo* RNA synthesis to firstly generate negative polarity RNA from the positive-stranded genomic RNA template (Ferrari and Huang, 2013; Selisko et al., 2006). The latter then serves as a template for synthesis of more RNA strands of positive polarity which are used for further protein translation or packaged to form infectious virions (Selisko et al., 2014). Like other polymerases, DENV RdRp possesses an architecture that is conserved across different classes and families of polymerases. This structure mimics a half-closed right hand with three sub-domains termed, “fingers”, “palm” and “thumb” (Sahili and Lescar, 2017; Zhao et al., 2015).

In this study, we explored the application of recombinant DENV2 NS5 antigen. Firstly, we constructed the DENV 2 NS5 RdRp domain and the full-length NS5 expression vectors, expressed and purified the recombinant NS5 (rNS5) C terminus 70 kDa (NS5-C70) and the full-length NS5 (104 kDa). Although the prokaryotic expression system is suitable for the expression of proteins with the molecular weight of 30–70 kDa, much smaller than that of NS5 (104 kDa), we successfully expressed the rNS5 after optimizing the codons of its full-length. By ELISA tests, we found that the two purified recombinant products reacted with patients' sera of all the four serotypes. Next, we compared the DENV 2 rNS5-based indirect ELISA for IgG detection with two commercially available dengue IgG indirect ELISA kits, Mybiosource and Panbio. The results suggest that DENV 2 rNS5 might be an effective antigen for the detection of DENV infection. Moreover, the rNS5-C70 can react with DENV 2 and 4 and may also be used as auxiliary antigen in diagnostics.

2. Materials and methods

2.1. Construction and expression of plasmid vector NS5-C70

The sequence of DENV 2 NS5 RdRp domain, approximately 2/3 of NS5 from C-terminal (residues 272–900, 70 kDa), was searched from GenBank accession number AF038403.1. DNA fragment of the NS5 RdRp domain was synthesized by Detai Biologics Company (Nanjing, China) with *Bam*H I site (5′-*GGATCC*-3′) added at the 5′ end, and *Hind* III site (5′-*AAGCTT*-3′) added at the 3′ end. Then it was inserted into pQE30 plasmid between restriction sites of *Bam*H I and *Hind* III after amplification with PCR kit (Takara, Beijing China) and restriction endonuclease (NEB, USA) digestion. The recombinant plasmid pQE30/NS5-70 was transformed into the chemically competent E. coli DH5α to be amplified. After sequence confirmation, pQE30/NS5-70 was transformed into the E. coli M15 (Dingguo, Beijing, China). The transformed cells were first grown in 5 mL LB culture, containing 50 μg/mL of Kanamycin (Kan) and 35 μg/mL of Ampicillin (Amp), at 37 °C overnight at 180 rpm, to test the expression and solubility of NS5 C-terminal 70 kDa (NS5-C70). Then one mL of cultured medium was transferred into 1 L LB culture containing 50 μg/mL of Kan and 35 μg/mL of Amp. The culture was incubated at 37 °C and rotated at 180 rpm. IPTG was added at a final concentration of 1 mM when the culture reached an absorbance at 600 nm (A600) of 0.6-1.0. The induced culture was then

rotated at 180 rpm for 4 h at 25 °C. After induction, the cells were harvested by centrifugation at 10,000 rpm for 15 min at 4 °C, then re-suspended in 60 mL lysis buffer containing 20 mM HEPES (pH 7.4), 1 M NaCl, 20 mM β-mercaptoethanol, 1.0 mg/mL lysozyme, and 1% PMSF, followed by sonication (Sonics, Connecticut, USA) on ice for 20–30 min (pulse on 15 s /off 15 s, 40% amplitude), and fractionated by centrifugation at 7800 × *g* for 1 h at 4 °C. The supernatants were kept for protein purification later.

2.2. Construction and expression of plasmid vector full-length NS5

The sequence of full-length DENV 2 NS5, about 900 amino acid residue with a molecular weight of 104 kDa, was searched from GenBank accession number AF038403.1. NS5 sequence was codon optimized using the MaxCodon™ Optimization Program for expression in *E. coli*. The full-length optimized DNA fragment was synthesized by Detai Biologics Company (Nanjing, China) with *Nde* I site (5′-*CATATG*-3′) added at the 5′ end and *Hind* III site (5′-*AAGCTT*-3′) added at the 3′ end. Then it was ligated into vector pET30a between restriction sites of *Nde* I and *Hind* III after amplification with PCR and digestion, and the recombinant plasmid pET30a/NS5 was transformed into the chemically competent *E. coli* Top 10. After sequence confirmation, pET30a/NS5 was transformed into *E. coli* BL21(DE3) and expressed as described previously, except that 50 μg/mL of Kanamycin was added and induced culture was grown at 15 °C overnight. After induction, cells were harvested by centrifugation at 10,000 × g for 15 min at 4 °C, and re-suspended in 60 mL lysis buffer containing 20 mM PB (pH 7.2), 150 mM NaCl with 1% triton X-100, 1 ug/mL pepstatin A, and 1 ug/mL leupeptin followed by sonication (Sonics, Connecticut USA) on ice for 20–30 min (pulse on 15 s /off 15 s, 40% amplitude), and fractionated by centrifugation at 12,000 × g for 1 h at 4 °C. The sediments were kept and washed with washing buffer containing 50 mM tris (pH 8.0), 150 mM NaCl with 1% triton X-100, 5 mM EDTA, and 2 mM DTT, then the inclusion bodies were dissolved in binding buffer containing 20 mM tris (pH 8.0), 150 mM NaCl, 8 M urea, 20 mM imidazole.

2.3. Western blot analysis

The rNS5 and rNS5-C70 were respectively separated on 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose filter (NC) membranes (Merck Millipore, Darmstadt, Germany). The membranes were then incubated in blocking buffer, 5% (w/v) skim milk in PBST, overnight at 4 °C. Mouse anti-His antibody (EarthOx, San Francisco, CA USA) and rabbit anti-NS5 antibody (Sigma-Aldrich), which were produced by immunization with synthetic peptides corresponding to amino acids 879–892 of NS5 in rabbits were used at a dilution of 1/5,000 and 1/1000 respectively in blocking buffer and incubated with the membranes for 3 h at room temperature. After washing with PBST 5 times over five minutes, the membranes were respectively incubated with goat anti-mouse and goat anti-rabbit secondary antibodies with DyLight 800 labeled (KPL, Maryland, USA) at a dilution of 1/10,000 for 1 h at room temperature in cassettes, and washed 5 times. Then the expressed rNS5 and rNS5-C70 were visualized by Odyssey Infrared Imaging (LI-COR, Lincoln, NE USA).

2.4. Purification of the rNS5 RdRp domain fragment

The NS5-C70 with a hexahistidine tag modifications was expressed in soluble form. The supernatants as previously described were filtered through a 0.45 μm filter and subjected to affinity chromatography using His Trap Ni-IDA agarose beads column (Detai, Nanjing, China) previously equilibrated with 20 mL basic buffer (0.5 M NaCl and 10% HEPES) of pH 7.4. The protein solution was added to the column and flowed down at a rate of 0.5–1 mL/min by controlling the sample quantity. After loading the expressed proteins, the column was washed gradually with increasing concentrations of imidazole (10, 20, and 40 mM in basic buffer). The target protein was then eluted with 250 mM imidazole in basic buffer and the eluted fractions were collected at various stages during the purification step, then analyzed on a 10% SDS-PAGE gel and visualized by Coomassie blue staining. Fractions containing the purified recombinant proteins were pooled and the proteins were concentrated using a 50 kD viva spin concentrator (Merck Millipore, Darmstadt, Germany) at 4000 × *g* for 15 min at 4 °C, and protein storage solution (PBS with 40–50% glycerol) was added 1: 1 to the concentrated protein solution. Purified products were quantified using the Bradford protein assay kit (Tiangen, Beijing, China).

2.5. Purification of the full-length rNS5

The full-length NS5 was expressed as inclusion body which was verified by Ni-IDA affinity chromatography. The inclusion bodies were washed with washing buffer containing 50 mM tris (pH 8.0), 150 mM NaCl with 1% Triton X-100, 5 mM EDTA, and 2 mM DTT, and the inclusion bodies were dissolved in binding buffer containing 20 mM Tris (pH 8.0), 500 mM NaCl, 8 M Urea, and 10 mM imidazole. At the same time, the Ni-IDA column was equilibrated with 20 mL binding buffer. The dissolved protein solution was added to the column and flowed down at a rate of 0.5–1 mL/min by controlling the sample quantity. After loading the expressed proteins, the column was washed gradually with increased concentrations of imidazole (10, 50, and 100 mM in binding buffer). Then the target protein was eluted with 500 mM imidazole in binding buffer. Each eluted fraction was collected for SDS-PAGE analysis. Samples containing the target protein were then pooled. The eluant containing target protein was then transferred into a 15 cm length dialysis bag after the bag was boiled in 500 mL 1 mM EDTA and 2% $NaHCO_3$ solution for 5 min, and in 500 mL 1 mM EDTA solution for another 5 min. The renatured NS5 was dialyzed in the buffer containing PBS (pH 7.4), 2 mM EDTA, 4 mM GSH, 0.4 mM GSSG, 2 mM EDTA, 0.4 M L-arginine, and 2 M urea overnight, and a further dialysis with PBS (pH 7.4) with 10% glycerol for about 6–8 h. Then the supernatant was filtered with a 0.45 μm filter, and stored −20 °C.

2.6. Reactivity analyses of recombinant products with sera of patients infected with DENV 1–4

Reactivity assay was conducted based on indirect ELISA to detect dengue-specific IgG in human sera using DENV 2 rNS5 and rNS5-C70 antigens. The rNS5 and rNS5-C70 were previously diluted with a coating solution (50 mM Na_2CO_3, pH 9.6) to a concentration of 1 μg/mL. Two 96-microwell plates were separately coated with rNS5 and rNS5-C70 at a volume of 100 μL (100 ng) per well and kept at 4 °C overnight. After the coating solution was poured, the plates were completely washed with PBS five times and pat dried using paper towels to remove residual liquid. The plates were filled with blocking buffer (5% skim milk in PBS) and incubated at 37℃ for 2 h. Sera were obtained from patients with laboratory-confirmed DENV 1, 2, 3, or 4 infections (The Prince of Wales Hospital, Hong Kong). Sera from healthy volunteers who took occupational medical examinations at the physical examination center in Chaoyang Hospital, Beijing, were used as controls. Volunteers with a history of visiting tropical areas were excluded from the study. Sera from patients and controls were diluted in two-fold falling dilutions from 1: 500 to 1: 16,000. Each sample was added at a volume of 100 μL/well to the coated plates and incubated at 37 °C for 2 h. After the solution was poured, the plates were washed with PBST and were pat dried using paper towels. Next, secondary antibody, namely, HRP-conjugated mouse anti-human IgG antibody (ZSGB-BIO, Beijing, China), which was diluted 1:1000 in blocking buffer, were added at a volume of 100 μL/well and incubated at 37 °C for 2 h. After washing the plates five times with PBST, tetramethylbenzidine (TMB) substrate (100 μL) was added to each well. Finally, the plates were incubated in the dark at 37℃ for 10 min for color development, and 100 μL of termination solution (2 M H_2SO_4) was

added to stop the reaction. Color intensity was measured at 450 nm using a microplate reader (PerkinElmer, Massachusetts USA). Statistical significance analyses were performed by one-way ANOVA analysis with GraphPad 6.0. Samples with A_{450} higher than 2.1 fold of the average of the control group were defined positive.

2.7. Comparison of the rNS5-based IgG indirect ELISA with two commercially available IgG ELISA kit

Serum samples (NO.1–50) were collected from patients at The Prince of Wales Hospital, who were not suspected with DF or related symptoms, these serum samples were the same as (Lee et al., 2018). The samples included 30 seropositive samples (No.1–30) and 20 seronegative samples (NO. 31–50) determined by a commercially available non-type-specific MyBioSource Dengue Virus IgG ELISA kit (MyBioSource, USA) (https://www.mybiosource.com) with the sera diluted at 1:21. Serum samples (NO.51–60) were collected from 10 dengue patients with laboratory-confirmed (Panbio Dengue IgM Capture ELISA kit) dengue infection clinical symptoms at The Prince of Wales Hospital. The aforementioned rNS5-based IgG indirect ELISA was performed for all 60 serum samples, which were diluted at 1:100 and 1:500. Another commercially available kit, namely, Panbio Dengue IgG Indirect ELISA kit (Panbio, Australia), was used for 45 serum samples (NO.6–50), which were diluted at 1:100 according to the manufacturer's instructions. The ELISA results from commercially available kits were used as reference to determine the efficacy of the rNS5-based ELISA.

3. Results

3.1. Expression and purification of the NS5-C70

The NS5 RdRp domain coding sequence was inserted in the expression vector pQE30, with a hexahistidine tagged at N-terminus. We obtained the 70 kDa rNS5-C70 in soluble form (Fig. 1A). Purification was conducted with His-Trap Ni-IDA column. The target protein was eluted in 250 mM imidazole solution as observed from the SDS-PAGE (Fig. 1A). After concentration with a 50 kDa Viva Spin Concentrator (Fig. 1B), we obtained 3.04 mg of purified fragment per liter of culture. Western blot analyses further confirmed that the NS5-C70 was reactive with anti-His antibody (Fig. 3A) and anti-NS5 antibody (Fig. 3C).

3.2. Expression and purification of the full-length NS5

DENV 2 NS 5 coding sequence was inserted in the expression vector pET30a. Unlike NS5-C70, the hexahistidine was the C-terminally tagged. It was found that the NS5 was expressed in inclusion bodies by His-Trap Ni-IDA affinity chromatography (Fig. 2A). Ni-IDA columns were used for protein purification after the inclusion bodies were dissolved, and the target protein was eluted in 500 mM imidazole solution as observed from the SDS-PAGE (Fig. 2B). The 104 kDa purified rNS5 was renatured by dialysis. The rNS5 was reactive with anti-His antibody (Fig. 3B) and anti-NS5 antibody (Fig. 3D) as shown by Western blot. The purified protein yield was 5.66 mg per liter of culture.

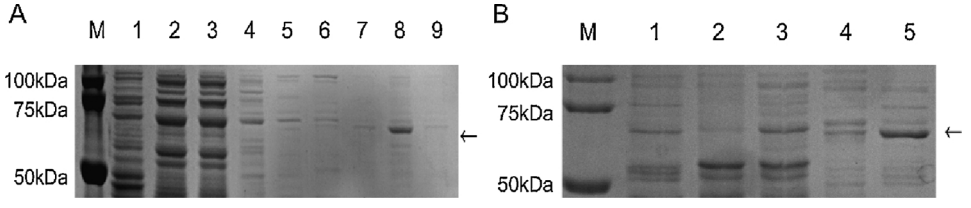

Fig. 1. SDS-PAGE analyses of recombinant NS5-C70. A: M, molecular weight. 1, supernatant from un-induced bacterial lysate. 2–3, supernatant from induced bacterial lysate. 4–9, elutions of 10 mM, 20 mM, 40 mM, 250 mM, 250 mM, and 250 mM imidazole solution after supernatant incubation with Ni-IDA. B: 1, bacterial proteins of pQE30 empty plasmid. 2, the supernatant from un-induced bacterial lysate. 3, supernatant from induced bacterial lysate. 4, protein purified by His Trap Ni-IDA. 5, purified proteins further concentrated by 50 kD Viva Spin Concentrator.

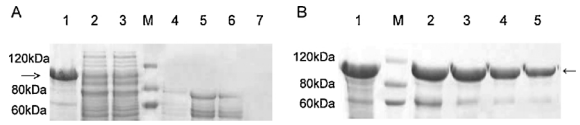

Fig. 2. SDS-PAGE analyses of recombinant NS5. A: 1, precipitate from induced bacterial lysate. 2, supernatant from induced bacterial lysate. 3, effluent after supernatant incubation with Ni-IDA. 4–7, elutions of 50 mM, 100 mM, 100 mM, and 500 mM imidazole solution. B: 1, effluent after disolved inclusion bodies incubation with Ni-IDA. 2–5, elutions of 50 mM, 100 mM, 500 mM and 500 mM imidazole solution.

3.3. Reactivity of recombinant products using sera of patients with DENV 1–4 infection

The results of DENV 2 rNS5- and rNS5-C70- based ELISA are shown in Fig. 4. All of the absorption values of DENV 2 rNS5 and rNS5-C70 reacting to sera from patients with dengue serotype 1–4 infection at different dilutions (1:500–1:16,000) were higher than those of reactions to the control group. However, rNS5-C70 reacted only with serotypes 2 and 4, with significantly higher absorption values than the control group. Meanwhile, rNS5 detected all of the four serotypes in each dilution (1: 500–1: 16,000), with significantly higher absorption values than the controls. The ratios of sample absorption values to the average OD of the control were ≥ 2.1 (Table 1).

3.4. Comparison of the rNS5-based IgG ELISA with two commercially available IgG ELISA Kits

We compared the developed rNS5-based IgG ELISA with two commercially available IgG indirect ELISA kits using 50 serum samples (Table 2). The positive agreement values of rNS5 assay with MyBioSource IgG and Panbio IgG assay were 83% (25/30) and 70% (14/20), respectively, and the positive agreement value of Panbio IgG with Mybiosource IgG was 56% (14/25). The Panbio IgG assay test results of the negative sera (NO. 31–50) determined by MyBioSource IgG assay, were all negative. The negative agreement values of rNS5 assay with MyBiosource IgG or Panbio IgG assay were 50% (10/20) and 48.4% (15/31), respectively (Table 3). In the 10 serum samples from dengue patients, who had been laboratory and clinically confirmed, the results of the rNS5 assay were positive for all (Table 4).

4. Discussion

Previous studies on DENV NS5 have mainly focused on its potential as a vaccine antigen or an anti-viral target, because it is the main aiming site of anti-DENV T cell-based immune response in human (Alves et al., 2016; Mladinich et al., 2012), and its RdRp activity plays a central role in viral genome replication (Steffens et al., 1999). However researches on NS5 in infection diagnosis are rarely reported, except that DENV 2 NS5 can be used to distinguish between DENV infection and other flaviviruses infections by ELISA, but in Narayan's study, NS5 was obtained by isolating viral proteins after culturing DENV 2 in Vero cells (Narayan et al., 2016), which was difficult to obtain with high experimental safety requirements and low yield. So far, there are few reports about DENV NS5 expression. In this study, full-length NS5 was

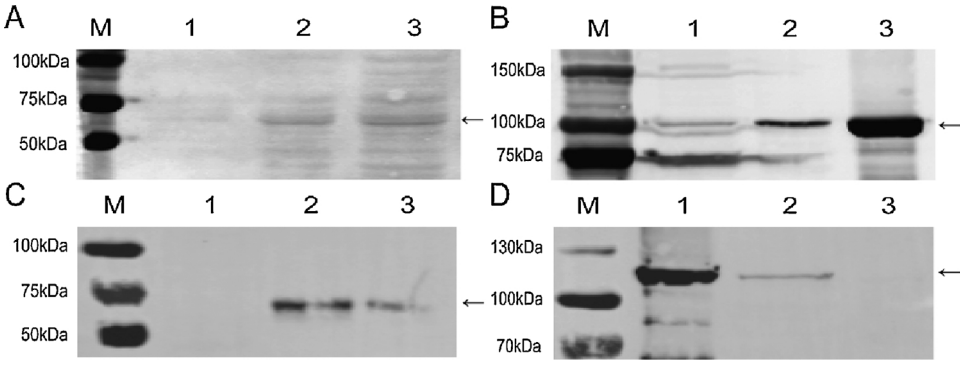

Fig. 3. Western blot analyses of recombinant NS5-C70 (A, C) and NS5 (B, D). A, B: anti-His as the primary antibody. C, D: anti-NS5 as the primary antibody. A: 1, bacterial proteins of pQE30 empty plasmid. 2, supernatant from un-induced bacterial lysate. 3, supernatant from induced bacterial lysate. B: 1, supernatant from bacterial lysate. 2, precipitate from bacterial lysate. 3, supernatant from dissolved inclusion bodies. C: 1, bacterial proteins of pQE30 empty plasmid. 2, supernatant from induced bacterial lysate. 3, supernatant from un-induced bacterial lysate. D: 1, dissolved inclusion bodies. 2, precipitate from bacterial lysate. 3, bacterial proteins of pET30a empty plasmid.

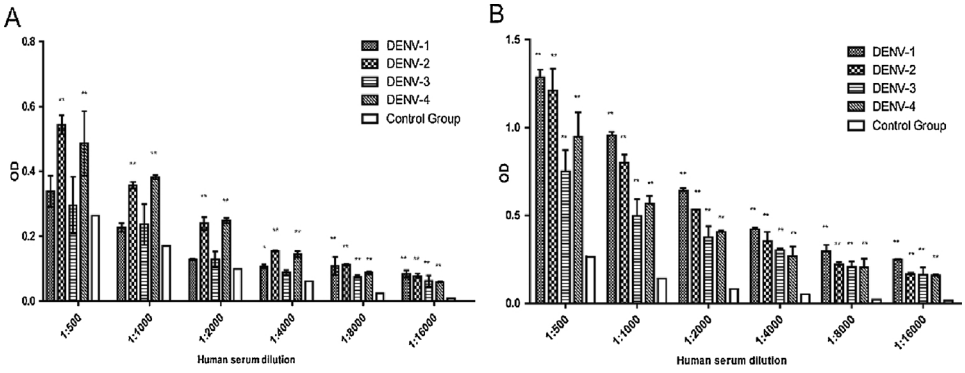

Fig. 4. Reactivity of serially diluted sera from four patients with confirmed dengue 1–4 infection against 100 ng of purified recombinant NS5-C70 (A) and NS5 (B) by ELISA. The control group was provided by healthy people and excludes those who have been to dengue epidemic regions. Statistical analyses were performed by one-way ANOVA tests, **, $P < 0.01$, *, $P < 0.05$.

successfully expressed through codon optimization and selection of host strains *E. coli* BL21(DE3), which will greatly reduce the cost and the cumbersome procedures.

Here we developed an rNS5-based ELISA for the detection of DENV IgG. DENV 2 rNS5 can react with the sera of patients who were positive for DENV 1, 2, 3 or 4, thereby suggesting that anti-NS5 antibodies come into being in the sera of patients with DENV infection, which is consistent with a recent report about the promising usage of NS5 as vaccine antigen on the basis of its reactivity with sera from patients with DENV 1–4 infection (Alves et al., 2016). Compared with the full-length DENV 2 rNS5, DENV 2 rNS5-C70 exhibited considerably decreased diagnostic efficacy and was only positively reacted with sera of type 2 and 4, probably because the DENV 4 NS5 RdRp domain shares higher sequence identity to DENV 2 than DENV 1 and 3 (Teramoto et al., 2017). Nevertheless, DENV 2 rNS5-C70 can also be used as an auxiliary diagnostic antigen.

Compared to the rNS5-based ELISA reported in this study, the antigens of the MyBioSource and Panbio kits were respectively a mix of E-proteins and NS1 of all four DENV serotypes. The positive agreement value of rNS5-based assay with either MyBioSource or Panbio assay was higher than that of the two commercial assays. The rNS5 assay had a lower negative agreement value with both commercially available kits. However, according to the performance characteristics of the Panbio IgG Indirect ELISA kit, its sensitivity to the detection of primary infection was only 33% (28/84) (Figure S1), indicating false negative results may have occurred with the commercial available kits. The rNS5 assay of the ten positive serum samples determined by Panbio Dengue IgM kit, were all positive. There are some published studies describing the cross-reactions of Panbio Dengue IgM with other flaviviruses

Table 2
Results of the 50 serum samples tested by rNS5-based IgG ELISA and two commercially available IgG ELISA kits. (MyBioSource Dengue Virus IgG ELISA kit and Panbio Dengue IgG Indirect ELISA).

MyBioSource IgG	Panbio IgG	rNS5	Numbers
+	+	+	14
+	NT	+	5
+	+	–	0
+	–	+	6
+	–	–	5
–	+	+	0
–	–	+	10
–	+	–	0
–	–	–	10
Total			50

NT: Not tested.

Table 1
ELISA results of rNS5-C70 and rNS5 reactions with dengue patients' sera (absorption ratio of patients to average controls).

Sera dilution	rNS5-C70				rNS5			
	DENV 1	DENV 2	DENV 3	DENV4	DENV 1	DENV 2	DENV 3	DENV4
1:500	1.3	2.1^{+}	1.1	1.8	4.9^{+}	4.6^{+}	2.9^{+}	3.6^{+}
1:1000	1.3	2.1^{+}	1.4	2.2^{+}	6.6^{+}	5.5^{+}	3.4^{+}	3.9^{+}
1:2000	1.3	2.4^{+}	1.3	2.5^{+}	8.7^{+}	7.2^{+}	5.1^{+}	5.5^{+}
1:4000	—	—	—	—	9.8^{+}	8.2^{+}	7.0^{+}	6.2^{+}
1:8000	—	—	—	—	16.4^{+}	12.3^{+}	11.5^{+}	11.4^{+}

— : Absorption values < 0.2, data did not have a reference value.
$^{+}$ Absorption value ratio of patients to average controls ≥ 2.1.

Table 3
Comparison of the rNS5-based IgG ELISA with two commercially available IgG ELISA Kits (MyBioSource Dengue Virus IgG ELISA kit and Panbio Dengue IgG Indirect ELISA).

	Positive agreement		Negative agreement	
Test methods	MyBioSource IgG	Panbio IgG	MyBioSource IgG	Panbio IgG
rNS5	83% (25/30)	70% (14/20)	50% (10/20)	48.4% (15/31)
Panbio IgG	56% (14/25)		100% (20/20)	

Table 4
ELISA results of rNS5 reactions with 10 dengue patients' serum samples (No.51–60).

Sera dilution	Sample NO.									
	51	52	53	54	55	56	57	58	59	60
1:100	2.5^{+}	2.8^{+}	3.0^{+}	4.9^{+}	2.1^{+}	3.2^{+}	4.9^{+}	4.4^{+}	4.9^{+}	3.0^{+}
1:500	2.5^{+}	2.1^{+}	1.9	3.8^{+}	1.7	3.2^{+}	7.8^{+}	6.9^{+}	8.1^{+}	2.1^{+}

All of the ten serum samples (NO.51–60) were collected from patients with laboratory-confirmed (Panbio IgM ELISA kit) and clinical symptoms of DENV infection.

$^{+}$ Absorption value ratio of patients to average controls ≥ 2.1.

(Ayukekbong et al., 2017; Blacksell et al., 2011; Hunsperger et al., 2014). Possible cross-reactions with antibodies to other flaviviruses would need to be more extensively tested with rNS5 in further studies.

The number of specific dengue positive sera tested may be limiting in this study. However, initial studies suggest that the rNS5-based ELISA can be used for preliminary screening of non-type-specific DENV infection, and can also be used to estimate the prevalence of DENV infection in a region and then determine whether to support implementation of the immunization program (Lee et al., 2018). More extensive studies with larger sample size from patients with each of the four DENV serotypes, as well as negative controls, and different geographical dengue endemic regions would be necessary to fully validate the rNS5-based ELISA.

Conflict of interests

The author declare that they have no competing interests. Paul KS Chan was not involved in the review process of this manuscript.

Acknowledgements

We thank Prof. Chang-Wen Ke of Guangdong Provincial Center of Disease Control and Prevention for his professional advice and instructive suggestions. This work was supported by Anti-viral Medicine Science and Technology, Beijing Municipal International Cooperation Base (015000546317517).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jviromet.2018.12.005.

References

Alves, R.P., Pereira, L., Fabris, D.L., Salvador, F.S., Santos, R.A., Zanotto, P.M., Romano, C.M., Amorim, J.H., Lc, D.S.F., 2016. Production of a recombinant Dengue virus 2 NS5 protein and the potential use as a vaccine antigen. Clin. Vac. Immunol. Cvi 23, 460.
Ayukekbong, J.A., Oyero, O.G., Nnukwu, S.E., Mesumbe, H.N., Fobisong, C.N., 2017. Value of routine dengue diagnosis in endemic countries. World J. Virol. 6, 9–16.
Bäck, A.T., Lundkvist, A., 2013. Dengue viruses – an overview. Infect. Ecol. Epidemiol. 3.
Balasubramanian, A., Teramoto, T., Kulkarni, A.A., Bhattacharjee, A.K., Padmanabhan, R., 2017. Antiviral activities of selected antimalarials against dengue virus type 2 and Zika virus. Antiviral Res. 137, 141–150.
Beltramello, M., Williams, K.L., Simmons, C.P., Macagno, A., Simonelli, L., Quyen, N.T.H., Sukupolvipetty, S., Navarrosanchez, E., Young, P.R., Silva, A.M.D., 2010. The human immune response to dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity. Cell Host Microbe 8, 271–283.
Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., Drake, J.M., Brownstein, J.S., Hoen, A.G., Sankoh, O., 2013. The global distribution and burden of dengue. Nature 496, 504–507.
Blacksell, S.D., Jarman, R.G., Bailey, M.S., Tanganuchitcharnchai, A., Jenjaroen, K., Gibbons, R.V., Paris, D.H., Premaratna, R., Silva, H.J.D., Lalloo, D.G., 2011. Evaluation of six commercial point-of-care tests for diagnosis of acute dengue infections: the need for combining NS1 antigen and IgM/IgG antibody detection to achieve acceptable levels of accuracy. Clin. Vac. Immunol. Cvi 18, 2095–2101.
Brady, O.J., Gething, P.W., Bhatt, S., Messina, J.P., Brownstein, J.S., Hoen, A.G., Moyes, C.L., Farlow, A.W., Scott, T.W., Hay, S.I., 2012. Refining the global spatial limits of dengue virus transmission by evidence-based consensus. PLoS Negl. Trop. Dis. 6 (8), e1760 (2012-8-7) 6.
Chow, V.T., Chan, Y.C., Yong, R., Lee, K.M., Lim, L.K., Chung, Y.K., Lamphua, S.G., Tan, B.T., 1998. Monitoring of dengue viruses in field-caught Aedes aegypti and Aedes albopictus mosquitoes by a type-specific polymerase chain reaction and cycle sequencing. Am. J. Trop. Med. Hyg. 58, 578–586.
Dejnirattisai, W., Jumnainsong, A., Onsirisakul, N., Fitton, P., Vasanawathana, S., Limpitikul, W., Puttikhunt, C., Edwards, C., Duangchinda, T., Supasa, S., 2010. Cross-reacting antibodies enhance dengue virus infection in humans. Science 328, 745–748.
Dussart, P., Labeau, B., Lagathu, G., Louis, P., Nunes, M.R., Rodrigues, S.G., Storckherrmann, C., Cesaire, R., Morvan, J., Flamand, M., 2006. Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. Clin. Vac. Immunol. Cvi 13, 1185.
Ebi, K.L., Nealon, J., 2016. Dengue in a changing climate. Environ. Res. 151, 115–123.
Ferrari, E., Huang, H.C., 2013. A Novel Hepatitis C Virus NS5B Polymerase Assay of De Novo Initiated RNA Synthesis Directed from a Heteropolymeric RNA Template. Humana Press.
Gelanew, T., Hunsperger, E., 2018. Development and characterization of serotype-specific monoclonal antibodies against the dengue virus-4 (DENV-4) non-structural protein (NS1). Virol. J. 15, 30.
Halstead, S.B., 2007. Dengue. Lancet 370, 1644–1652.
Halstead, S.B., Russell, P.K., 2016. Protective and immunological behavior of chimeric yellow fever dengue vaccine. Vaccine 34, 1643–1647.
Hermann, L.L., Thaisomboonsuk, B., Poolpanichupatam, Y., Jarman, R.G., Kalayanarooj, S., Nisalak, A., Yoon, I.K., Fernandez, S., 2014. Evaluation of a dengue NS1 antigen detection assay sensitivity and specificity for the diagnosis of acute dengue virus infection. PLoS Negl. Trop. Dis. 8, e3193.
Hunsperger, E.A., Yoksan, S., Buchy, P., Nguyen, V.C., Sekaran, S.D., Enria, D.A., Vazquez, S., Cartozian, E., Pelegrino, J.L., Artsob, H., 2014. Evaluation of commercially available diagnostic tests for the detection of dengue virus NS1 antigen and anti-dengue virus IgM antibody. PLoS Negl. Trop. Dis. 8, e3171.
Johnson, B.W., Russell, B.J., Lanciotti, R.S., 2005. Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR assay. J. Clin. Microbiol. 43, 4977–4983.
Koonin, E.V., 1991. The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. J. Gen. Virol. 72 (Pt 9), 2197.
Koonin, E.V., 1993. Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and lambda 2 protein of reovirus. J. Gen. Virol. 74 (Pt 4), 733–740.
Kuhn, R.J., Zhang, W., Rossmann, M.G., Pletnev, S.V., Corver, J., Lenches, E., Jones, C.T., Mukhopadhyay, S., Chipman, P.R., Strauss, E.G., 2002. Structure of dengue virus. Cell 108, 717–725.
Kumarasamy, V., Wahab, A.H.A., Chua, S.K., Hassan, Z., Chem, Y.K., Mohamad, M., Chua, K.B., 2007. Evaluation of a commercial dengue NS1 antigen-capture ELISA for laboratory diagnosis of acute dengue virus infection. J. Virol. Methods 140, 75–79.
Lee, P., Yeung, A.C., Chen, Z., Chan, M.C., Sze, K.H., Chan, P.K., 2018. Age-specific seroprevalence of dengue infection in Hong Kong. J. Med. Virol.
Lim, S.P., Noble, C.G., Shi, P.Y., 2015. The dengue virus NS5 protein as a target for drug discovery. Antiviral Res. 119, 57.
Lu, G., Gong, P., 2013. Crystal Structure of the full-length Japanese encephalitis virus NS5 reveals a conserved methyltransferase-polymerase interface. PLoS Pathog. 9, e1003549.
Mackenzie, J., 2010. Wrapping things up about virus RNA replication. Traffic 6, 967–977.
Makino, Y., Tadano, M., Saito, M., Maneekarn, N., Sittisombut, N., Sirisanthana, V., Poneprasert, B., Fukunaga, T., 1994. Studies on serological cross-reaction in sequential flavivirus infections. Microbiol. Immunol. 38, 951–955.
Mladinich, K.M., Piaskowski, S.M., Rudersdorf, R., Eernisse, C.M., Weisgrau, K.L., Martins, M.A., Furlott, J.R., Partidos, C.D., Brewoo, J.N., Osorio, J.E., 2012. Dengue virus-specific CD4^{+} and CD8^{+} T lymphocytes target NS1, NS3 and NS5 in infected Indian rhesus macaques. Immunogenetics 64, 111–121.
Mustafa, M.S., Rasotgi, V., Jain, S., Gupta, V., 2015. Discovery of fifth serotype of dengue virus (DENV-5): a new public health dilemma in dengue control. Med. J. Armed Forces India 71, 67–70.
Narayan, R., Raja, S., Kumar, S., Sambasivam, M., Jagadeesan, R., Arunagiri, K., Krishnasamy, K., Palani, G., 2016. A novel indirect ELISA for diagnosis of dengue fever. Indian J. Med. Res. 144, 128–133.
Normile, D., 2013. Tropical medicine. Surprising new dengue virus throws a spanner in disease control efforts. Science 342, 415.

Pal, S., Dauner, A.L., Mitra, I., Forshey, B.M., Garcia, P., Morrison, A.C., Halsey, E.S., Kochel, T.J., Wu, S.J.L., 2014. Evaluation of dengue NS1 antigen rapid tests and ELISA kits using clinical samples. PLoS One 9, e113411.
Paul, D., Bartenschlager, R., 2015. Flaviviridae replication organelles: oh, what a tangled web we weave. Annu. Rev. Virol. 2, 289.
Peeling, R.W., Artsob, H., Pelegrino, J.L., Buchy, P., Cardosa, M.J., Devi, S., Enria, D.A., Farrar, J., Gubler, D.J., Guzman, M.G., 2010. Evaluation of diagnostic tests: dengue. Nat. Rev. Microbiol. 8, S30.
Rosen, L., Gubler, D., 1974. The use of mosquitoes to detect and propagate dengue viruses. Am. J. Trop. Med. Hyg. 23, 1153.
Sabchareon, A., 2015. Efficacy and long-term safety of a dengue vaccine in regions of endemic disease. N. Engl. J. Med. 373, 1195–1206.
Sahili, A.E., Lescar, J., 2017. Dengue virus non-structural protein 5. Viruses 9, 1–20.
Scott, L.J., 2016. Tetravalent dengue vaccine: a review in the prevention of dengue disease. Drugs 76, 1301.
Selisko, B., Dutartre, H., Guillemot, J.C., Debarnot, C., Benarroch, D., Khromykh, A., Desprès, P., Egloff, M.P., Canard, B., 2006. Comparative mechanistic studies of de novo RNA synthesis by flavivirus RNA-dependent RNA polymerases. Virology 351, 145–158.
Selisko, B., Wang, C., Harris, E., Canard, B., 2014. Regulation of Flavivirus RNA synthesis and replication. Curr. Opin. Virol. 9, 74.
Shu, P.Y., Huang, J.H., 2004. Current advances in dengue diagnosis. Clin. Diagn. Lab. Immunol. 11, 642.
Singh, M.V., Weber, E.A., Singh, V.B., Stirpe, N.E., Maggirwar, S.B., 2017. Preventive and therapeutic challenges in combating Zika virus infection: are we getting any closer? J. Neurovirol. 23, 1–11.
Sophia, G., Anna, S., Dayan, G.H., John, J., Melanie, S., Diane, V.D.V., Anh, W.T., 2016. Safety overview of a recombinant live-attenuated tetravalent dengue vaccine: pooled analysis of data from 18 clinical trials. PLoS Negl. Trop. Dis. 10, e0004821.
Steffens, S., Thiel, H.J., Behrens, S.E., 1999. The RNA-dependent RNA polymerises of different members of the family Flaviviridae exhibit similar properties in vitro. J. Gen. Virol. 80 (Pt 10), 2583–2590.
Stephenson, J.R., 2005. Understanding dengue pathogenesis: implications for vaccine design. Bull. World Health Organ. 83, 308–314.
Taylor-Robinson, A., 2016. A putative fifth serotype of dengue-potential implications for diagnosis. Ther. Vac. Des.
Teramoto, T., Balasubramanian, A., Choi, K.H., Padmanabhan, R., 2017. Serotype-specific interactions among functional domains of Dengue virus 2 non-structural proteins (NS) 5 and NS3 are crucial for viral RNA replication. J. Biol. Chem. 292.
Vicente, G., Pablo, G.V., Vicente, M., Antonio, E.J., 2016. Looking for inhibitors of the dengue virus NS5 RNA-dependent RNA-polymerase using a molecular docking approach. Drug Des. Devel. Ther. 10, 3163–3181.
Wichmann, O., Vannice, K., Asturias, E.J., De, E.A.L., Longini, I., Lopez, A.L., Smith, P.G., Tissera, H., Yoon, I.K., Hombach, J., 2017. Live-attenuated tetravalent dengue vaccines: the needs and challenges of post-licensure evaluation of vaccine safety and effectiveness. Vaccine.
Wong, S.J., Boyle RHDemarest, V.L., Woodmansee, A.N., Kramer, L.D., Li, H., Drebot, M., Koski, R.A., Fikrig, E., Martin, D.A., Shi, P.Y., 2003. Immunoassay targeting nonstructural protein 5 to differentiate West Nile virus infection from dengue and St. Louis encephalitis virus infections and from flavivirus vaccination. J. Clin. Microbiol. 41, 4217.
Wu, S.J.L., Lee, E.M., Putvatana, R., Shurtliff, R.N., Porter, K.R., Suharyono, W., Watts, D.M., King, C.C., Murphy, G.S., Hayes, C.G., 2001. Detection of dengue viral RNA using a nucleic acid sequence-based amplification assay. J. Clin. Microbiol. 39, 2794.
Zhao, Y., Soh, T.S., Zheng, J., Chan, K.W., Phoo, W.W., Lee, C.C., Tay, M.Y., Swaminathan, K., Cornvik, T.C., Lim, S.P., 2015. A crystal structure of the dengue virus NS5 protein reveals a novel inter-domain interface essential for protein flexibility and virus replication. PLoS Pathog. 11, e1004682.