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Short communication

Identification and molecular characterization of human antibody fragments specific for dengue NS5 protein

Yongqian Zhao[a,b,1], Nicole J. Moreland[a,1,2], Moon Y.F. Tay[a], Chin Chin Lee[a], Kunchithapadam Swaminathan[b,c], Subhash G. Vasudevan[a,b,*]

[a] Program in Emerging Infectious Diseases, DUKE-NUS Graduate Medical School, 8 College Road, Singapore 169857, Singapore
[b] NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, 28 Medical Drive, Singapore 117456, Singapore
[c] Department of Biological Sciences, National University of Singapore, Singapore 117543, Singapore

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ABSTRACT

The multifunctional dengue nonstructural (NS) protein 5 from the four serotypes of dengue virus (DENV1–4) is essential for viral replication and harbors a methyl transferase (MTase) and a RNA-dependent RNA-polymerase domain (RdRp). There are limited comparative studies of NS5 from the four DENV serotypes and this is further hampered by a lack of cross-reactive NS5 antibodies. In this study, recombinant NS5 proteins were expressed, purified, enzymatically characterized, and used strategically as bait in biopanning experiments with a naïve human Fab phage-display library to identify serotype specific or cross-reactive Fab fragments. Using a combination of peptide competition ELISA and peptide phage display the epitopes of the cross-reactive Fabs were mapped to the first alpha helix of the MTase domain (5M1) and the priming loop of the RdRp domain (5R3). The epitope of a third, serotype-specific Fab (5M3) was mapped to aa19–30 of the DENV3 MTase domain. Together the recombinant proteins and specific antibodies will facilitate further mechanistic studies of the DENV replication complex.

Dengue virus (DENV), a mosquito-transmitted member of the *Flaviviridae* family, consists of four antigenically distinct serotypes (DENV1–4) and is an infection threat to nearly 40% of the world's population (Bhatt et al., 2013). The DENV genome is an ~11 kb positive strand RNA comprising a single open reading frame (ORF). This encodes a single polyprotein that is co- and post-translationally processed by both host cellular proteases and the DENV viral protease (NS2B/NS3) into three structural (Capsid, Membrane, Envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Lescar et al., 2008; Lindenbach et al., 2007).

The replication of dengue genome takes place in the ER-membrane associated replication complexes (RC) (Mackenzie, 2005; Salonen et al., 2005) with two viral non-structural (NS) proteins, NS3 and NS5, residing in the functional center. NS3 possesses protease activity (with cofactor NS2B) for polyprotein cleavage, helicase activity for unwinding dsRNA preceeding replication, and NTPase/RTPase activities essential for genome capping (Bartelma and Padmanabhan, 2002; Benarroch et al., 2004; Erbel et al., 2006; Li et al., 2005; Luo et al., 2010; Sampath et al., 2006; Yon et al., 2005). NS5 also harbors multiple enzymatic activities: RNA-dependent RNA polymerase (RdRp) activity required for genome replication and methyltransferase (MTase)/guanylyltransferase (GTase) activities for genome capping (Egloff et al., 2002; Issur et al., 2009; Kroschewski et al., 2008; Ray et al., 2006; Selisko et al., 2010; Yap et al., 2007; You and Padmanabhan, 1999). Other viral nonstructural proteins (NS1, NS4A, NS4B, etc.) (Lindenbach and Rice, 1999; Miller and Krijnse-Locker, 2008; Rathore et al., 2011; Umareddy et al., 2006) and host proteins such as those involved in the ubiquitin proteasome pathway, NF90, eEF1A (Fernandez-Garcia et al., 2011; Gomila et al., 2011) are also reported to constitute replication complex, but the complete depiction of the RC remains elusive. NS5 has recently been shown to have sub-cellular localization patterns that differ among the four DENV serotypes (Tay et al., 2013). It also acts as an interferon response antagonist via NS5 mediated STAT2 binding and degradation (Ashour et al., 2009; Mazzon et al., 2009). The importance of NS5 in viral replication and host immune response modulation makes it an excellent target for developing

Abbreviations: DENV, dengue virus; NS, non-structural protein; NS5FL, full-length NS5 protein; MTase, methyltrasferase; RdRp, RNA-dependent RNA polymerase; Fab, antigen-binding fragment.

* Corresponding author at: Program in Emerging Infectious Diseases, DUKE-NUS Graduate Medical School, 8 College Road, Singapore 169857, Singapore.
Tel.: +65 65166718; fax: +65 62212529.
E-mail addresses: subhash.vasudevan@duke-nus.edu.sg, subhash.vasudevan@gmail.com (S.G. Vasudevan).
[1] These authors contributed equally to this work.
[2] Present address: School of Biological Sciences, University of Auckland, New Zealand.

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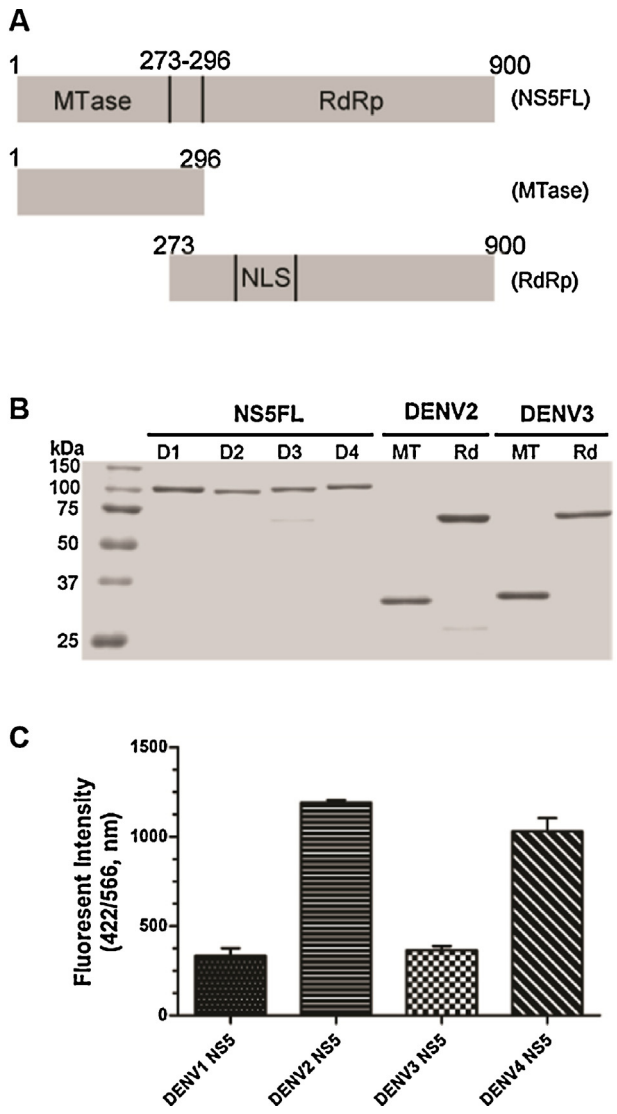

Fig. 1. Expression and purification of NS5 proteins. (A) NS5 recombinant constructs used in this work. (B) Purified recombinant NS5 constructs were resolved by 12% SDS-PAGE and stained with Comassie blue. (Lanes 1–4) Full length NS5 proteins: 1, DENV1 (EU081230.1); 2, DENV2 (M29095); 3, DENV3 (AY662691) and 4, DENV4 (GQ398256). (Lane 5–8) MTase domain (MT) and RdRp domain (Rd) from DENV2 and DENV3 as indiated. (C) Comparison of NS5FL polymerase activity monitored by amount of released BBT, excitation 422 nm/emission 566 nm. DENV2 and 4 NS5 proteins show significantly higher activity than NS5 proteins of DENV1 (28%) and DENV3 (31%) ($P < 0.005$ for DENV2 and $P < 0.05$ for DENV4).

anti-dengue inhibitors. Monoclonal antibodies with specificity to NS5 are valuable tools to study the various functions and interactions of NS5, and to resolve the replication complex.

In this study a naïve human Fab-phage library was screened using phage antibody technology to identify NS5 specific antibody fragments. A number of NS5 protein variants were prepared as antigens for biopanning and characterization. Recombinant MTase (aa1–296) and RdRp (aa273–900) domains of NS5 from DENV2 and DENV3 were expressed and purified from *Escherichia Coli* as previously described (Kroschewski et al., 2008; Yap et al., 2007). In addition, full length NS5 proteins (NS5FL; DENV1, aa1–899; DENV2–4, aa1–900) from the four serotypes (DENV1, EU081230; DENV2, M29095; DENV3, AY662691; and DENV4, GQ398256) were cloned, expressed and purified from *E. coli* using standard techniques (Fig. 1A). Polymerase activity of the full-length NS5 proteins was assessed using an in vitro assay containing 50 nM RNA template and 2 μM of the substrate BBT-ATP (Niyomrattanakit et al., 2011). Interestingly NS5FL polymerase activities of DENV and 4 were similar, and higher than that observed for NS5 from DENV1 and 3 (Fig. 1B). This correlates with the phylogenetic relationship of the viruses, with the NS5 protein sequences of DENV2 and 4 being more closely related than DENV1 and 3 NS5 (Vasilakis and Weaver, 2008).

Initial biopanning experiments were performed with the MTase and RdRp domains from DENV3 due to their greater stability in solution compared with full-length NS5 proteins. DENV3 MTase and RdRp were immobilized onto streptavidin magnetic beads following biotinylation and used as bait for panning against a naïve human Fab-phage library (Moreland et al., 2010). Six unique clones with specificity for MTase and RdRp were identified, but Fab-phage ELISAs with NS5 proteins from DENV1–4 revealed all of these clones were serotype specific for DENV3 (Table 1). Biopanning was repeated using proteins from alternating DENV serotype in screening rounds (DENV3 followed by DENV2) to enrich for cross-reactive clones. This strategy resulted in the identification of two further clones (5M1 and 5R3) that were cross-reactive for NS5 proteins from DENV1–4 in Fab-phage ELISA (Table 1). Sequence analysis of the eight encoded Fab with IMGT/V-QUEST revealed that all of the Fabs specific for NS5 MTase comprise of variable heavy chain (VH) sequences from VH3 and variable light chain (VL) sequences from Vλ6 family. Sequences for Fabs with specificity for NS5 RdRp are more varied being derived from three VH families (VH1, VH3 and VH4) and three VL families (Vλ1, Vκ2, Vκ3). The CDR3 regions for all clones show some degree of diversity in length.

To express and purify the NS5 Fabs, the phagemids of each of the unique clones were digested with *SalI* to remove the phage gene III sequence as described (Moreland et al., 2010). Fabs were expressed with a hexa-histidine and *c-myc* tag at the C-terminus, and immunoassays were performed with an HRP conjugated anti-*c-myc* antibody, to further characterize the two cross-reactive Fabs (5M1 and 5R3) and one of the DENV3 specific Fabs (5M3). 5M1 could detect purified NS5FL from all four serotypes as well as MTase domains of DENV2 and 3 by Western blot following reducing SDS-PAGE (Fig. 2Ai). It was also able to detect NS5 from DENV1–4 infected Huh-7 cell lysates by Western blot (data not shown). An ELISA against NS5FL proteins from DENV1–4 showed a concentration dependent binding curve for all four serotypes (Fig. 2Aii). Taken together, these results show 5M1 binds a linear epitope on MTase conserved across DENV1–4. In contrast, the Western blots and ELISA for 5M3 show this Fab is specific DENV3 as it only detects DENV3 NS5 proteins (Fig. 2Ci and ii). The RdRp specific Fab 5R3 failed to detect purified NS5 or dengue infected Huh-7 cell lysate by Western blot (data not shown). However, it was able to recognize native RdRp of DENV2 and 3 in a dot blot (Fig. 2Bi) and NS5FL from DENV1–4 in an ELISA (Fig. 2Bi and ii). This suggests 5R3 recognizes a conformational epitope in the NS5 RdRp domain that is conserved across the four serotypes.

In order to map the binding sites for two cross-reactive antibodies 5M1 and 5R3, peptide competition ELISAs were performed using an array of overlapping 15-mer peptides spanning DENV2 NS5FL protein (178 peptides in total). The peptide corresponding to DENV2 NS5 aa6–20 blocked binding of 5M1 to the MTase coated plate, while the remaining peptides showed no competition (Fig. 3Ai). Residues 6–20 form the first α-helix in MTase domain of NS5 protein and a sequence alignment of this region for the four DENV serotypes shows the residues are conserved, which is consistent with the cross-reactivity of 5M1 (Fig. 3Aii). Similarly, the peptide corresponding to DENV2 NS5 residues 786–800 strongly reduced the binding of 5R3 to the DENV2 RdRp coated on the plate, while the remaining peptides showed no competition (Fig. 3Bi). Residues 786–800 are within the priming loop (aa782–809) of the RdRp domain, which binds the template strand during replication. The crystal structure of DENV3 RdRp revealed a number of