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Production of a Versatile Pan-DENV NS5 Polyclonal Antibody and Structural Epitope Analysis of Immunogen Performance

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Abstract

Background: Dengue is a major mosquito-borne viral disease worldwide, with approximately 400 million infections reported annually and no fully effective antiviral treatments available. The non-structural protein NS5 is a promising antiviral target due to its critical role in viral RNA replication and host interactions. Although commercial monoclonal and polyclonal anti-NS5 antibodies are available, none were found to combine broad reactivity against all four dengue virus serotypes with suitability for Western Blot (WB), Immunofluorescence (IF), and Immunoprecipitation (IP) assays. This study aimed to develop and functionally characterize a versatile polyclonal antibody targeting a conserved region of DENV NS5 for immunodetection applications.

Methods: Conserved recombinant regions from the methyltransferase (MTase) and RNA-dependent RNA polymerase (RdRp) domains of DENV2 NS5 were expressed in *Escherichia coli* and used as immunogens in a murine model.

Results: Both recombinant proteins exhibited predicted antigenic potential, but only the anti-NS5-RdRp antibody efficiently recognized native NS5 in infected cells. The resulting antibody detected NS5 from all four dengue serotypes in WB, with lower nonspecific background than a widely used commercial antibody. In addition, it proved effective in IF and IP assays in DENV1- and DENV2-infected cells, enabling co-immunoprecipitation of NS3, a known NS5 interactor. Retrospective structural epitope analyses suggested that the RdRp immunogen contained surface-accessible conformational features more compatible with antibody recognition in the native protein context.

Conclusion: These findings identify the anti-NS5-RdRp antibody as a valuable tool for dengue research and illustrate how structural epitope analyses can provide insights into differences in immunogen performance.

Key words: Dengue virus, Epitopes, Recombinant proteins
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Introduction

Dengue Virus (DENV) is the most widespread arboviral threat, placing nearly 50% of the global population at risk of infection. Annual incidence exceeds 400 million cases, with endemic transmission confirmed in over 100 countries across tropical and subtropical regions [1]. Epidemiological analyses reveal a concerning 85% increase in reported cases between 1990 (30.7 million) and 2019 (56.9 million), accompanied by a 28% rise in mortality [2,3]. This expansion is associated with climate change, urbanization, and adaptive shifts in vector populations.

DENV, a member of the Flavivirus genus, comprises four antigenically distinct serotypes (DENV1 to DENV4). Its ~11 *kb* positive-sense single-stranded RNA genome encodes a ~3000-amino acid polyprotein, which is co- and post-translationally cleaved into three structural proteins (capsid, pre-membrane, and envelope) and seven non-structural proteins (NS1,

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NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [4,5]. Among these, NS5 (~105 *kDa*, 900 residues) is the most conserved, showing 92-96% amino acid identity across serotypes. Its N-terminal domain harbors methyltransferase activity (MTase), while the C-terminal domain functions as an RNA-dependent RNA polymerase (RdRp), both essential for viral replication [6,7].

In addition to its enzymatic functions, NS5 interacts with multiple host pathways. *In silico* analyses suggest that human NS5-interacting partners are involved in JAK-STAT signaling, RNA processing, cell cycle progression, necroptosis, protein synthesis, and ER-mediated protein processing, among other biological activities [8]. These characteristics make NS5 an attractive target for pan-serotype antiviral and diagnostic strategies [9,10]. Its high level of conservation also positions it as a promising diagnostic marker, further supporting the development of polyclonal antibodies for both research and clinical applications.

However, currently available commercial anti-NS5 antibodies present important limitations, including narrow serotype specificity (primarily DENV2) and limited compatibility across key experimental platforms such as WB, IF, and IP (Supplementary tables S1 and S2). This methodological gap may have contributed to a research bias in the field, with most published studies on NS5 focusing predominantly on DENV2.

This study aimed to develop and characterize a polyclonal antibody targeting a conserved region of the DENV NS5 protein with cross-reactivity against all four serotypes and compatibility with multiple immunodetection platforms, including WB, IF, and IP assays. Given the central role of NS5 in viral replication and several host pathways, such a reagent could facilitate investigations into dengue virus biology and virus–host interactions. In addition, recent dengue vaccine strategies increasingly incorporate non-structural proteins, including NS5, to promote broader cellular immune responses and cross-serotype protection [11]. Therefore, a well-characterized anti-NS5 antibody may also support future evaluations of NS5 expression and immune responses in vaccine-related contexts.

Materials and Methods

In silico assessment of the DENV-NS5 immunogenicity

To identify regions with the highest potential to induce an immune response, antigenic determinants of the NS5 protein from DENV1, DENV2, DENV3, and DENV4 (NCBI database accession numbers NP_722465.1, NP_739590.2, YP_001531176.2, and NP_740325.1, respectively) were identified using the Predicted Antigenic Peptides program with default parameters (http://imed.med.ucm.es/Tools/antigenic.pl), which implements the method described by Kolaskar and Tongaonkar (1990) [12].

B-cell epitope prediction was subsequently performed using the IEDB Analysis Resource (version 2.16; https://tools.iedb.org). Both the linear epitope prediction tools and the DiscoTope conformational epitope prediction tool were run using the default prediction parameters and cutoff values implemented in the platform. Linear epitope prediction incorporated physicochemical properties associated with antigenicity, including β-turn propensity [13], surface accessibility [14], flexibility [15], and hydrophilicity [16]. Conformational epitopes were predicted with DiscoTope using the DENV2 NS5 structure available in the Protein Data Bank (PDB ID: 6KR2), integrating residue spatial organization and structural features derived from antibody–antigen complexes resolved by X-ray crystallography [17].

DENV2 ns5 gene cloning

The DENV2 *ns5* gene sequence (NC_001474.2) was used to design the primers Fwd-NS5$^{160-232}$ (catatgCGAACACTCAGAGTCCTC) and Rev- NS5$^{160-232}$ (ggatccTAACACTGATGACACTATG), which amplify a fragment corresponding to amino acids residues 160 to 232 in the MTase domain of DENV2 NS5. Similarly, the primers Fwd-NS5$^{661-770}$ (catatgAGTGGAGGAGATGATTGTGTTGTT) and Rev-NS5$^{661-770}$ (ggatccTAAACGTCTGTGGAAGT) were design to amplify a region corresponding to residues 661 to 770 within the RdRp domain. Lowercase letters indicate the recognition sites for the restriction enzymes *NdeI* and *BamHI*. Primers were designed using the "Primer Designer" program V.1.01 (http://www.scied.com/pr_cmpro.htm) and synthesized by Macrogen (Korea). Simultaneously, viral RNA was extracted from the supernatants of ATCC C6/36 cell cultures infected with DENV2 (isolate INS-2009) using the QIAamp Viral RNA® kit (Qiagen). The cDNA synthesis reaction was performed with MMLV retrotranscriptase using random primers (Promega) [18]. The NS5 PCR was conducted with Taq Platinum DNA polymerase (Invitrogen). The amplification protocol program comprised an initial denaturation of 95°*C* (3 *min*) and 35 cycles of denaturation at 94°*C* (30 *s*), annealing at 50°*C* (30 *s*) and extension at 72°*C* (30 *s*), followed by a final extension step at 72°*C* (5 *min*).

PCR products were purified using the Wizard® SV Gel & PCR Clean-Up System kit (Promega), cloned into the pJET1.2/blunt vector (Thermo Scientific), and subsequently subcloned into the pMAL-c5X expression vector (NEB) following the manufacturers' instructions. The resulting constructs (NS5-MTase-pMAL and NS5- RdRp-pMAL) were used to transform NEB Express *Escherichia coli* (*E. coli*) cells. Transformant colonies were screened by PCR using pMAL-c5X-specific primers, and the identity of the inserts was confirmed by DNA sequencing. The theoretical molecular weights of the recombinant NS5 fusion proteins were calculated using the Compute pI/Mw tool available at the ExPASy server (https://web.expasy.org/computepi/).

Expression and purification of the recombinant NS5-MBP proteins

The expression of the recombinant NS5-MTase-MBP and NS5-RdRp-MBP proteins (hereafter rNS5-MTase and rNS5-RdRp) was conducted in 200 *ml* of LB medium (Luria Bertani, Sigma) supplemented with ampicillin (100 *μg/ml*, Amresco) at 37°*C* with shaking (200 *rpm*). A one-milliliter sample was collected at the time of IPTG addition and at 1, 2, 3, and 4 *hr* post-induction (hpi) to monitor the expression of recombinant proteins by SDS-PAGE. The entire remaining culture was centrifuged at 4,000×*g* for 20 *min* at 4°*C*. The resulting cell pellet was then resuspended in lysis buffer (5 *mM* imidazole, 0.5 M NaCl, 10% Glycerol, 20 *mM* Tris-HCl pH=7.9, 1% Triton X-100, and protease inhibitor cocktail) and sonicated. The lysate was centrifuged at 18,000×*g* for 20 *min* at 4°*C*, and the supernatant and pellet were subjected to SDS-PAGE analysis to determine the solubility of the recombinant NS5-MBP. rNS5-MTase and rNS5-RdRp purification was carried out by affinity chromatography using amylose resin (NEB) following the manufacturer's instructions. Fractions eluted from the affinity chromatography were analyzed by SDS-PAGE and quantified by Bradford. The purity of the recombinant proteins was subsequently estimated by densitometric analysis of Coomassie Brilliant Blue-stained gels. Images were acquired using a ChemiDoc MP Imaging System (Bio-Rad) under non-saturating exposure conditions and analyzed using ImageJ software (v1.51J8, NIH, USA). Protein purity was calculated as the percentage of the integrated density of the recombinant protein band relative to the total integrated density of all protein bands within the same lane.

Polyclonal antibody production and purification

Purified rNS5-MTase and rNS5-RdRp were each intraperitoneally inoculated into two 4-week-old male Wistar rats. Two additional rats served as non-immunized controls (control serum). Anti NS5-DENV polyclonal antibodies were purified from the immune sera as described previously [18]. Briefly, affinity purification was performed by eluting antibodies specifically bound to their corresponding recombinant antigens immobilized on nitrocellulose membranes following transfer from SDS-polyacrylamide gels [18]. The purified antibodies were designated as anti-NS5-MTase and anti-NS5-RdRp.

Cell culture and DENV infection

Isolates from the four serotypes of DENV were used to infect BHK-21 cells. DENV1 (strain D1OMS) and DENV3 (strain D3OMS) were donated by the "Programa de Estudio y Control de Enfermedades Tropicales" (PECET) (Universidad de Antioquia, Medellín, Colombia). DENV2 (isolate INS-2009) and DENV4 (isolate D4INS2016) were donated by the Virology Laboratory at the "Instituto Nacional de Salud" (Bogotá, Colombia). BHK-21 cells (ATCC® CCL-10TM) were cultured in Dulbecco's modified Eagle's medium (DMEM. Sigma) supplemented with 10% Bovine Fetal Serum (BFS) (Hyclone) at 37°*C* in 5% CO_2 environment. When the cell monolayer reached 80% confluence, infection with DENV was done at MOI (multiplicity of infection) 1 for one *hr*. Then, the cells were maintained in DMEM-SFB 2% for 48 *hr*. Subsequently, infected BHK-21 cells were treated with lysis solution (50 *mM* Tris HCl pH=7.5, 100 *mM* NaCl, 0.1% Triton X-100, 0.5 *mM* PMSF, and 1 *mM* protease inhibitors) and sonicated. Proteins were quantified using the Bicinchoninic acid method and stored at -70°*C* until use. To confirm DENV infection, a WB assay was performed using the anti-$NS3^{403\text{-}599}$ antibody, which was produced in a previous study [18].

Western blotting assays on BHK-21 cells infected with DENV

Protein extracts from DENV-infected BHK-21 cells were used for WB analysis. Briefly, 30 *μg* of total protein per lane were separated by SDS-PAGE and transferred onto PVDF membranes (Thermo Scientific). Membranes were blocked with TBST containing 5% nonfat dry milk for 4 *hr* at room temperature and then incubated overnight at 4°*C* with serum from immunized rats (1:1000), purified anti-NS5-MTase or NS5-RdRp antibodies (1:1000), or a commercial anti-NS5 antibody (1:1000) (GTX124253; GeneTex). After washing, membranes were incubated with HRP-conjugated secondary antibodies (Thermo Scientific, 31470 or 31460, 1:5000). Signals were developed using DAB (Sigma) or chemiluminescent substrate detection (SuperSignal™ West Pico PLUS, Thermo Scientific). β-actin was used as a loading control and detected using an anti-actin antibody (Sigma, A5316) (1:10,000) and an HRP-conjugated anti-mouse secondary antibody (Thermo Scientific, 31430) (1:10,000).

Densitometric analysis of Western blots

All densitometric analyses were performed using ImageJ software (v1.51J8, NIH, USA). Two complementary analyses were performed. First, the Signal-to-Background Ratio (SBR) was calculated as (band intensity − background intensity)/background intensity, where band intensity corresponded to the mean pixel intensity within the protein band and background intensity to the mean pixel intensity measured in an adjacent region of identical size within the same lane. To compare NS5 signal intensity among DENV serotypes, a fixed-size region of interest was defined around the NS5 band and the corresponding β-actin band in each lane containing lysates from cells infected with DENV-1 to DENV-4. After subtraction of the surrounding background signal, the integrated density of each band was measured. To correct for lane-to-lane loading variation, NS5 integrated density was normalized to that of the corresponding β-actin band.

Immunofluorescence assays on BHK-21 cells infected with DENV

BHK-21 cells were seeded on round glass coverslips and infected with DENV2 following the infection protocol described above. Coverslips were then incubated overnight at 4°*C* with anti-NS5-RdRp antibody (1: 500), or with a commercial anti-NS5 antibody (GTX-124253; GeneTex) (1:1000). Subsequently, slides were incubated with the corresponding biotinylated secondary antibody (anti-rat, 1:2500; or anti-rabbit, 1:5000). After washing with PBS, slides were incubated with Alexa Fluor 594-conjugated streptavidin (Molecular Probes), and nuclei were counterstained with Hoechst 33342 (Sigma). Coverslips were mounted on microscope slides using Vectashield mounting medium (Vector Laboratories) and visualized using a Zeiss AxioImager A2 microscope equipped with an X-Cite 120Q fluorescence illumination system and ZEN 2012 software. Control experiments included infected cells incubated with control serum (1:250) and uninfected cells incubated with the anti-NS5-RdRp antibody. In addition, BHK-21 cells infected with DENV1 were also analyzed.

Immunoprecipitation assays

Protein extracts from DENV1- or DENV2-infected BHK-21 cells were used for IP assays with the anti-NS5-RdRp antibody. Briefly, 20 μl of anti-NS5-RdRp antibody was incubated with 1 *mg* of total protein extract for 4 *hr* on ice with gentle agitation. Protein A/G agarose beads (Thermo Scientific) were then added, and samples were incubated overnight at 4°*C* with shaking. The following day, samples were centrifuged, and the beads were washed and resuspended in Laemmli buffer for subsequent WB analysis. Control experiments included incubation with control serum or without antibody. In addition, NS5 immunoprecipitates were analyzed by WB using the anti-NS3 antibody to evaluate the previously reported interaction between NS3 and NS5 proteins.

Biological and technical replicates

Western blot experiments were independently repeated at least three times using independent biological samples. Immunofluorescence assays were performed using two independent biological replicates, each analyzed in three technical replicates. Immunoprecipitation experiments were independently repeated twice using independent biological samples. Representative images shown in the figures correspond to experiments displaying consistent results across independent replicates.

Results

Antigenicity analysis

The antigenicity analysis was performed to identify two independent NS5 regions for antibody generation, one within the MTase domain and another within the RdRp domain, while avoiding the region previously implicated in the NS3-NS5 interaction (NS5 residues 320-341). Analysis of NS5 sequences from the four DENV serotypes using the Predicted Antigenic Peptides program with default parameters identified the highest predicted antigenic peaks within the MTase domain approximately between residues 100 and 225. Similarly, in the RdRp domain, the highest peaks were located approximately between residues 660 and 775 (Supplementary figure S1A). Based on these results and primer design constraints, the regions selected for recombinant protein production and antibody generation corresponded to residues 160-232 and 661-770. Both regions displayed prominent antigenic peaks across the four DENV serotypes and shared predicted antigenic determinants (Supplementary Figure S1B).

Cloning, expression, and purification of recombinant NS5 proteins

Two fragments of the DENV2 *ns5* gene were amplified by RT-PCR: a 234-*bp* fragment corresponding to amino acid residues 160-232 within the MTase domain, and a 345-bp fragment corresponding to residues 661-770 within the RdRp domain of the NS5 protein. Both fragments were cloned into the pJET1.2 vector and subsequently subcloned into the pMAL-c5X expression vector. Sanger sequencing confirmed the identity of the cloned fragments. Following transformation and IPTG induction of *E. coli* NEB express cells, the NS5-MBP fusions proteins were detected by SDS-PAGE at the expected molecular weights (52 *kDa* and 56.57 *kDa* for rNS5-MTase and rNS5-RdRp, respectively) in both soluble and insoluble fractions (Figures 1A and 1C).

Subsequently, transformed bacterial cultures were lysed and centrifuged, and the resulting supernatants were subjected to affinity chromatography for purification of the NS5-MBP fusion proteins. SDS-PAGE analysis of the eluted fractions revealed bands of the expected molecular weight, together with a faint band at approximately 42 *kDa* corresponding to the MBP tag (Figures 1B and 1D). Soluble expression of the rNS5-MTase and rNS5-RdRp proteins yielded 4.0 *mg* and 2.2 *mg* of purified protein per 200 *ml* culture, respectively, corresponding to approximately 20 *mg/L* and 11 *mg/L*. These yields are higher than those previously reported for recombinant DENV NS5 proteins, which typically range from 2 to 8 *mg/L* (19-21). Densitometric analysis of Coomassie Brilliant Blue-stained gels from three independent protein preparations indicated that the recombinant NS5-MTase and NS5-RdRp proteins had mean purities of 89.7±1.4% and 92.1±0.8% (mean±SD, n=3), respectively. No prominent contaminating protein bands were detected, indicating that both recombinant proteins were obtained with a high degree of purity.

Evaluation of the anti-NS5-MTase and the anti-NS5-RdRp antibodies: western blot assays

Based on the SDS-PAGE results, selected chroma-

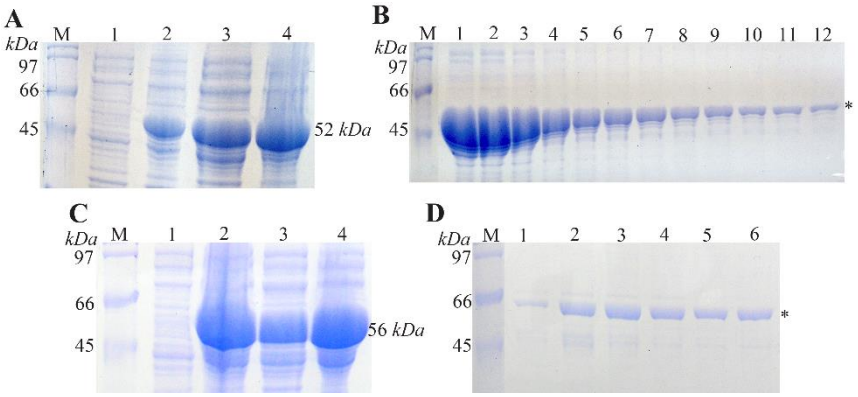

Figure 1. Expression, solubility, and purification of recombinant NS5–MBP fusion proteins.
SDS-PAGE (10%) stained with Coomassie Brilliant Blue. (A, C) IPTG-induced expression and solubility analysis of rNS5-MTase (A) and rNS5-RdRp (C). Lane 1: pre-induction (0 h). Lane 2: 4 *hr* post-induction. Lane 3: soluble fraction. Lane 4: insoluble fraction. (B, D) Affinity purification of rNS5-MTase (B) and rNS5-RdRp (D) using amylose resin. In (B), lanes 1–12 correspond to eluted fractions 1–12; in (D), lanes 1–6 correspond to eluted fractions 1–6. M: molecular weight marker (SDS6H2, Sigma). The asterisk indicates the expected recombinant NS5–MBP fusion proteins (rNS5-MTase: ~52 *kDa*; rNS5-RdRp: ~56 *kDa*).

tographic fractions were pooled and used for rat immunization. Following completion of the immunization protocol, the animals were bled, and approximately 6 *ml* of serum was collected from each rat. Initial WB analyses using the corresponding recombinant proteins demonstrated that sera from rats immunized with either rNS5-MTase or rNS5-RdRp specifically recognized their respective antigens. Similar results were obtained with the affinity-purified antibodies (hereinafter referred to as the anti-NS5-MTase antibody and anti-NS5-RdRp antibody). The crude sera and purified antibodies were subsequently evaluated by WB using protein extracts from DENV2-infected BHK-21 cells. Although both the crude serum from rats immunized with rNS5-MTase and the corresponding affinity-purified anti-NS5-MTase antibody recognized the recombinant antigen, no specific signal corresponding to endogenous NS5 was detected in infected-cell extracts under any of the WB conditions tested (Supplementary Figure S2). In contrast, both the crude serum and the affinity-purified anti-NS5-RdRp antibody successfully detected NS5 in extracts from DENV2-infected cells and were therefore selected for further characterization. Figure 2A shows the immunodetection of NS5 (~105 *kDa*) in DENV2-infected cell extracts using serum from a rat immunized with rNS5-RdRp, alongside the commercial anti-NS5 antibody GTX124253 (GeneTex). No signal was detected in extracts from uninfected cells or when serum from a non-immunized control rat was used as the primary antibody. Additionally, to compare the initial performance of the crude immune serum with that of a commercially available antibody, SBRs were determined by densitometric analysis of DAB-developed WBs from three independent experiments. The crude immune serum yielded a mean SBR of 1.54±0,47, whereas the commercial antibody yielded a mean SBR of 1.46±0,39 (mean±SD, n=3). These results indicate that the immune serum detected NS5 with signal-to-background performance similar to that of the commercial antibody. These quantitative measurements were used for descriptive purposes and were not intended to establish statistically significant differences between the antibodies.

To assess the reactivity of the anti-NS5-RdRp antibody against all four dengue virus serotypes, WB analyses were performed using extracts from BHK-21 cells infected with DENV1, DENV2, DENV3, or DENV4. Successful infection and extract quality were confirmed by detection of the NS3 protein using a previously characterized anti-NS3 antibody [18]. The anti-NS3 antibody detected NS3 in all four serotypes, with a slightly weaker signal observed in DENV2-infected cell extracts (Figure 2B). Similarly, the anti-NS5-RdRp antibody recognized NS5 in extracts from all four dengue virus serotypes, although signal intensity was reduced in DENV3-infected cells (Figure 2C). Densitometric analysis of chemiluminescent WBs from three independent experiments confirmed these observations. Mean signal-to-background ratios were 8.95±2.83, 2.64±1.08, 0.97±0.43, and 5.14±1.81 for DENV1, DENV2, DENV3, and DENV4, respectively, whereas non-infected cell extracts showed only background signal (0.09±0.04; mean±SD, n=3). In addition, normalization of NS5 band intensities to the corresponding β-actin loading control in two independent experiments yielded the same overall pattern, with mean NS5/β-actin ratios of 0.90 (DENV1), 0.71 (DENV2), 0.11 (DENV3), and 0.33 (DENV4), confirming that NS5 detection was consistently lowest in DENV3-infected cells. No viral proteins were detected when control serum was used as the primary antibody (Figure 2D).

Additional in silico analyses

To further characterize the antigenic properties of the selected NS5 recombinant regions, additional *in silico* analyses were performed to predict linear and

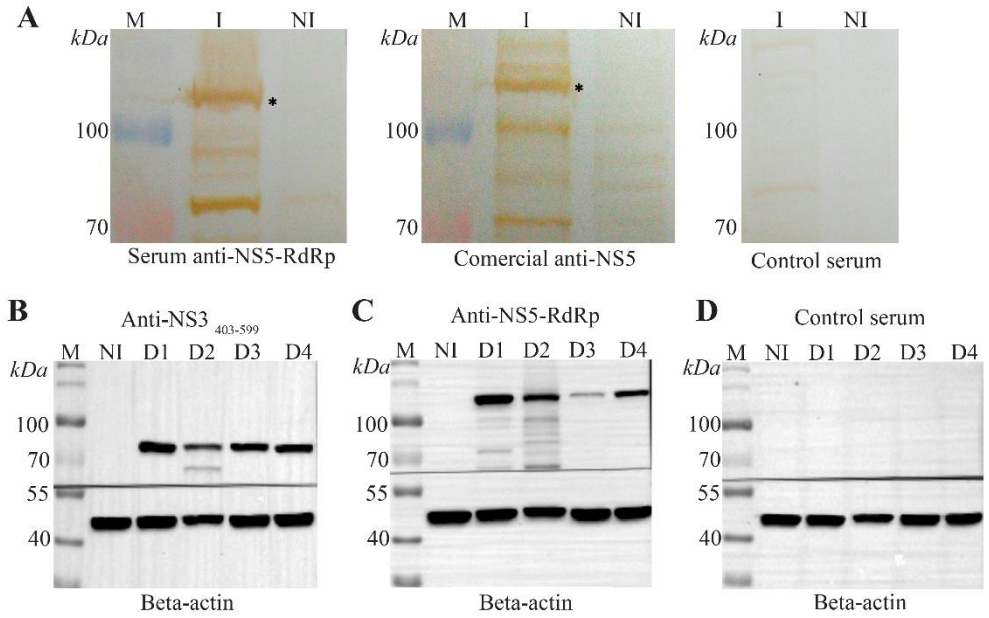

Figure 2. Immunodetection of DENV NS5 protein in infected BHK-21 cells.
A) Western blot analysis of DENV2-infected BHK-21 cell extracts using crude anti-NS5-RdRp rat serum, a commercial anti-NS5 antibody (GeneTex), and control serum. I: 30 *µg* of DENV2-infected BHK-21 cell extract; NI: 30 *µg* of uninfected BHK-21 cell extract. The asterisk indicates the viral NS5 protein (~105 *kDa*). Membranes were developed using DAB and H_2O_2. (B–D) Chemiluminescent WB analyses. Membranes were sectioned horizontally at approximately 55 *kDa*. The upper section was incubated with antiviral or control serum, whereas the lower section was probed with an anti-β-actin antibody as a loading control (~43 *kDa*). NI: uninfected BHK-21 cells; D1–D4: cells infected with DENV1, DENV2, DENV3, and DENV4, respectively. (B) Detection with anti-NS3 serum. (C) Detection with purified anti-NS5-RdRp antibody. (D) Detection with control serum. M: molecular weight marker (PageRuler Plus, Fermentas). Crude anti-NS5-RdRp serum, purified anti-NS5-RdRp antibody, anti-NS3 serum, commercial anti-NS5 antibody, and control serum were used at a dilution of 1:1,000. Anti-β-actin antibody was used at a dilution of 1:10,000.

conformational B-cell epitopes. Linear epitope prediction incorporated physicochemical parameters commonly associated with antigenicity, including hydrophilicity, flexibility, surface accessibility, and beta-turn propensity. Both recombinant regions contained multiple predicted linear B-cell epitopes supported to varying degrees by these complementary parameters (Table 1). Within the MTase region, predicted antigenic segments showed partial overlap among the evaluated features, whereas several epitopes within the RdRp region exhibited broader agreement across the different analyses. In particular, the SGDDCVVKP sequence displayed consistent associations with hydrophilicity, flexibility, surface accessibility, and beta-turn propensity. Nevertheless, the overall linear epitope analyses suggested that both recombinant regions contained antigenic determinants with the potential to elicit antibody responses.

To assess whether these regions might be accessible in the native NS5 structure, conformational B-cell epitope prediction was performed using DiscoTope. The MTase recombinant region (residues 160-232) was characterized predominantly by highly negative DiscoTope scores and residues with elevated structural contact numbers, suggesting limited surface accessibility in the folded protein (Table S3). Although a short segment spanning residues 209-215 displayed relatively increased propensity values, no extended conformational cluster with favorable DiscoTope scores was identified within this region. In contrast, the RdRp recombinant region contained a cluster of contiguous residues between positions 688 and 702 with comparatively higher propensity values, lower contact numbers, and DiscoTope scores closer to zero. Together, these features are consistent with the presence of a surface-accessible conformational patch within the native NS5 structure. Additional regions within the RdRp fragment showed lower predicted accessibility and less favorable conformational scores.

Taken together, these analyses suggest that both recombinant regions contained predicted linear antigenic determinants, consistent with their ability to induce antibody responses against the recombinant proteins. However, the presence of a predicted surface-accessible conformational patch within the RdRp region may offer a potential structural explanation for the superior recognition of native NS5 observed experimentally with the anti-RdRp antibody.

Evaluation of anti-NS5-RdRp antibody: IF assays

Following infection of BHK-21 cells with DENV1 or DENV2, NS5 protein localization was assessed by IF. As shown in figure 3, the anti-NS5-RdRp antibody localized NS5 predominantly to the nuclei of DENV2-infected cells, with intense, discrete punctate structures characteristic of Promyelocytic Leukemia Nuclear Bodies (PML-NBs). No signal was detected in unin-

Table 1. Predicted linear B-cell epitopes and associated physicochemical features within the NS5 recombinant regions

Recombinant region	Linear B-cell epitopes (Kolaskar., 1990)	Hydrophilicity	Flexibility	Accessibility	Turns
Mtase 160-169	RTLRVLNLVE	-	RTLRV, RTLR	-	VE, E
Mtase 176-192	TQFCIK-VLNPYMPSVIE	-	TQFC, TQF, TQ, T	T, TQ, NPYMPS	TQ, TQF, TQFC, TQFCI, VLNPYMP, LNPYMPS, NPYMPSV, PYMPSVI
Mtase 201-208	YGGALVRN	YGGA	Y, YGG, YGGA, YG-GAL, ALVRN, LVRN, VRN, RN, N	Y, YG, YGG, YGGA, RN, N	YGG, YGGA, YGGAL, YGGALV, GGALVRN, GALVRN, VRN, RN, N
Mtase 225-233	SGNIVSSVN	SGNI, SGN	SGNI		SGNI, SGN, SG
RdRp 661-669	SGDDCVVKP	SGDDCVV, GDDCVVK	SGDDCVV, CVVKP, VVKP, VKP, KP, P	VKP, KP, P	SGDDCV, SGDDCVV, GDDCVVK, DDCVVKP, VVKP, KP, P
RdRp 704-715	TQVPFCSHHFHE		TQV, TQVP	TQV, TQ	T, TQ, TQV, TQVP, TQVPFCS, QVPFCSH, PFCSHHF
RdRp 720-728	DGRVLVVPC	VPC, C	DGR, DGRV, DGRVL, DGRVLV, VPC, PC, C	C	VVPC, VPC, C
RdRp 749-756	RETACLGK		RETA, RETAC, CLGK, LGK	RETA, GK	R, RET, TACLGK, ACLGK, CLGK, LGK, GK

Linear B-cell epitopes were identified using the Kolaskar and Tongaonkar antigenicity prediction method. Additional analyses were performed using hydrophilicity, flexibility, surface accessibility, and beta-turn propensity algorithms available through the IEDB Analysis Resource. For each predicted epitope, overlapping amino acid segments identified by the corresponding physicochemical parameter are shown. The extent of overlap was used as an indicator of concordance among the different prediction methods.

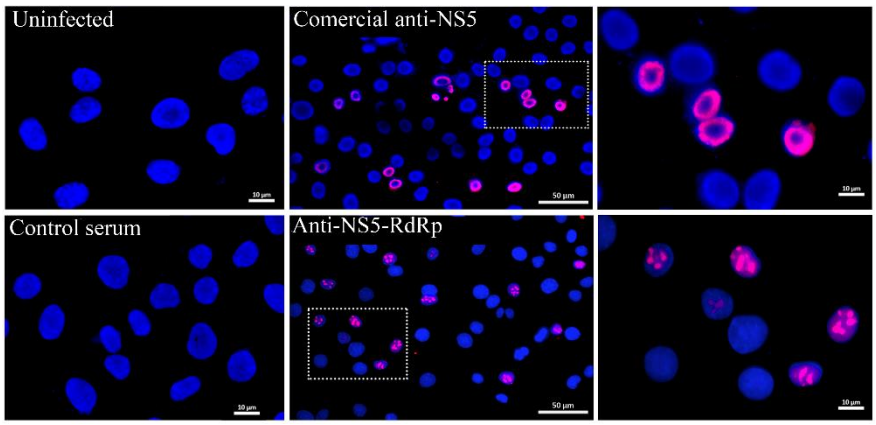

Figure 3. Immunofluorescence detection of DENV2 NS5 protein in BHK-21 cells.
Uninfected: non-infected BHK-21 cells incubated with the anti-NS5-RdRp antibody (1:500). Control serum: DENV2-infected BHK-21 cells incubated with serum from a non-immunized control rat (1:250). Commercial anti-NS5: DENV2-infected BHK-21 cells incubated with a commercial anti-NS5 antibody (GeneTex; 1:1000). Anti-NS5-RdRp: DENV2-infected BHK-21 cells incubated with the anti-NS5-RdRp antibody (1:500). Scale bars: 50 μm (low magnification) and 10 μm (high magnification).

fected or infected cells when control serum was used as the primary antibody (Figure 3). The commercial anti-NS5 antibody (GTX124253) also recognized nuclear NS5, though it exhibited a more intense perinuclear staining pattern. In contrast, in DENV1-infected BHK-21 cells, the anti-NS5-RdRp antibody localized NS5 mainly in the cytoplasm (Supplementary Figure S3A).

Evaluation of anti-NS5-RdRp antibody: Immunoprecipitation assays

The NS5 protein was successfully immunoprecipitated from DENV2-infected cell extracts using the anti-NS5-RdRp antibody (Figure 4A). In control reactions performed without antibody, the NS5 signal was detected predominantly in the unbound fraction, confirming the specificity of the immunoprecipitation procedure (Figure 4B). Given previous reports describing a direct interaction between NS3 and NS5 in DENV [22], immunoprecipitates were further analyzed by WB using a previously characterized anti-NS3 antibody. As shown in figure 4C, a band of approximately 70 *kDa* corresponding to NS3 was detected in the NS5 immunoprecipitates, indicating co-immunoprecipitation of the two viral proteins. Similar results were obtained using extracts from DENV1-infected BHK-21 cells (Supplementary Figure S3B). These results suggest that the anti-NS5-RdRp antibody is suitable for immunoprecipitation-based applications and can be used to recover NS5-containing protein complexes from infected cells. The detection of co-immunoprecipitated NS3 further supports its utility for studying protein

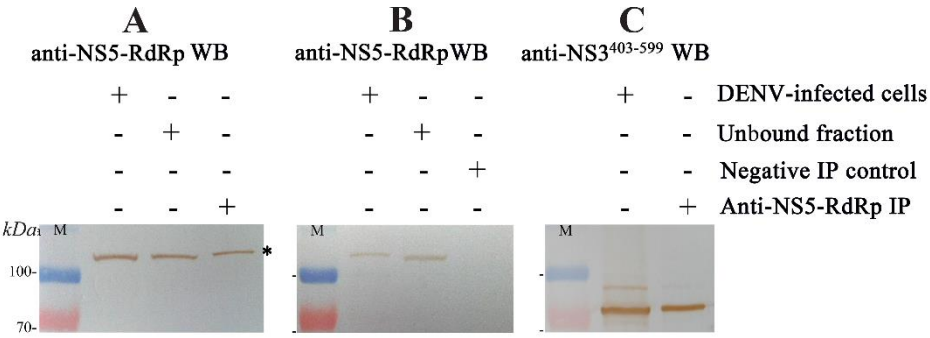

Figure 4. Immunoprecipitation (IP) assay using the anti-NS5-RdRp antibody in DENV2-infected BHK-21 cells.
(A) IP followed by WB detection with the anti-NS5-RdRp antibody. (B) Negative control in which IP was performed without antibody and analyzed by WB with the anti-NS5-RdRp antibody. (C) IP performed with the anti-NS5-RdRp antibody and analyzed by WB using an anti-NS3 antibody. All IP assays were carried out using 1 mg of total protein extract from DENV2-infected BHK-21 cells. The asterisk indicates the NS5 protein band (~105 *kDa*). M, molecular weight marker (PageRuler, Thermo Scientific).

interactions involving NS5 during dengue virus infection.

Discussion

The authors developed a pan-serotype rat-derived polyclonal antibody targeting a conserved, highly immunogenic region of DENV NS5. Pan-serotype recognition was demonstrated by WB against all four DENV serotypes, whereas the antibody was validated for IF and IP in DENV1- and DENV2-infected cells.

The present review of commercially available anti-NS5 antibodies suggested that the number of distinct antibodies may be considerably lower than the number of commercially available products. Although nine polyclonal and seven monoclonal antibodies were initially identified, several appeared to represent the same antibody marketed under different catalog numbers or by different suppliers (Supplementary Tables S1 and S2). Furthermore, few studies have systematically evaluated the performance of commercial anti-NS5 antibodies across multiple applications, particularly WB and IF. Among the polyclonal antibodies identified, the comparator selected for this study was chosen because it met two important criteria: reported recognition of NS5 from all four DENV serotypes by WB and published evidence supporting its use in both WB and IF. Likewise, evidence supporting the use of anti-NS5 antibodies for IP remains scarce and is currently limited to a single commercially available monoclonal antibody [19]. Together, these findings highlight the need for well-characterized anti-NS5 antibodies that have been functionally validated across multiple experimental applications.

In the present study, two conserved recombinant NS5 regions corresponding to the MTase and RdRp domains were evaluated as immunogens. Although both recombinant proteins elicited antibody responses, only the anti-NS5-RdRp efficiently recognized native NS5 in DENV-infected cells and was therefore selected for further characterization. This observation suggested that factors beyond sequence conservation and predicted linear antigenicity may influence the ability of recombinant immunogens to generate antibodies recognizing native NS5. To investigate this possibility, we performed retrospective epitope analyses combining sequence- and structure-based prediction methods. Linear B-cell epitope predictions suggested that both recombinant regions contained multiple antigenic determinants, consistent with their ability to induce antibody responses against the recombinant proteins. Therefore, differences in overall linear antigenicity alone do not appear sufficient to explain the contrasting experimental performance of the resulting antibodies. In contrast, conformational epitope prediction revealed notable differences between the two regions. The MTase fragment was largely composed of residues with unfavorable accessibility scores and high structural contact numbers, whereas the RdRp fragment contained a cluster of residues predicted to form a surface-accessible conformational patch in the context of the folded NS5 protein.

The increasing availability of highly accurate protein structure predictions generated by artificial intelligence methods, particularly AlphaFold and related approaches [23], has substantially expanded the use of structure-based analyses for immunogen design. Together with structure-based epitope prediction tools such as DiscoTope, these advances have expanded immunogen design beyond conventional sequence-based approaches by enabling the evaluation of structural features, including surface accessibility and conformational epitope distribution. Although these computational approaches do not replace experimental validation, they provide a valuable framework for prioritizing candidate immunogens and interpreting differences in antibody performance. In the present study, the retrospective structural analyses support this concept by providing a potential explanation for the superior performance of the NS5-RdRp immunogen compared with the NS5-MTase region, despite both regions

exhibiting favorable linear antigenicity predictions. These observations suggest that integrating structural accessibility and conformational epitope prediction with conventional linear antigenicity analyses may improve the rational selection of recombinant immunogens in future studies [24].

NS5 detection was similar between the crude anti-NS5-RdRp serum and the commercial GTX124253 antibody under the conditions evaluated. Notably, however, more background bands were observed with the commercial antibody than with the crude immune serum.

Following affinity purification, the anti-NS5-RdRp antibody retained strong NS5 detection while reducing nonspecific signals, resulting in a markedly cleaner WB profile than either the crude serum or the commercial antibody. Furthermore, the purified antibody successfully detected NS5 from all four DENV serotypes, with no detectable signal in uninfected control cells.

For IF assays, we considered the well-documented serotype-specific differences in NS5 subcellular localization. NS5 from DENV1 is primarily cytoplasmic, whereas in DENV2 and DENV3 it is predominantly nuclear. In DENV4, localization may vary between cytoplasmic and nuclear depending on the viral strain [25,26]. To reflect this variability, DENV1 and DENV2 were selected as representative models of cytoplasmic and nuclear NS5 localization, respectively. As expected, the anti-NS5-RdRp antibody detected predominantly cytoplasmic NS5 in DENV1-infected cells. In DENV2-infected cells, both the commercial GTX-124253 and the anti-NS5-RdRp antibodies revealed nuclear localization of NS5. Notably, however, the two antibodies produced distinct staining patterns: GTX-124253 showed stronger labeling at the nuclear periphery, whereas the anti-NS5-RdRp antibody generated a more pronounced punctate nuclear signal. Although the identity of these structures was not experimentally assessed, their appearance was reminiscent of PML nuclear bodies (PML-NBs). Previous studies have shown that all four DENV serotypes induce PML-NB disassembly and that nuclear-localized NS5 is sufficient to mediate this effect [27]. These observations support an antiviral role for PML-NBs during DENV infection and highlight NS5 as an important viral factor involved in counteracting host antiviral defenses. Together, these findings further reinforce the relevance of NS5 as a target for dengue research and antiviral development.

As mentioned above, only one monoclonal antibody has been experimentally shown to immunoprecipitate native NS5 from DENV2-infected cell lysates [19]. The choice between monoclonal and polyclonal antibodies can substantially influence IP performance. Because polyclonal antibodies recognize multiple epitopes on the target protein, they often provide more efficient antigen capture, particularly when some epitopes may be partially inaccessible in the native protein. In contrast, monoclonal antibodies offer high specificity but depend on the accessibility of a single epitope. Consistent with these considerations, the anti-NS5-RdRp antibody successfully immunoprecipitated NS5 from lysates of BHK-21 cells infected with either DENV1 or DENV2. Furthermore, it co-immunoprecipitated NS3, a well-established NS5-interacting partner whose interaction with NS5 involves residues 566-585 of NS3 and 320–341 of NS5 [22,28]. These results indicate that the antibody efficiently recognizes native NS5 while preserving physiologically relevant protein-protein interactions, supporting its utility for studies aimed at characterizing NS5-containing replication complexes, identifying novel NS5-interacting proteins, and investigating host-virus interaction networks.

The anti-NS5-RdRp antibody detected NS5 in WB assays across all four DENV serotypes and was further validated for IF and IP applications in DENV1- and DENV2-infected cells. Notably, it produced fewer nonspecific bands than a widely used commercial anti-NS5 antibody while showing similar NS5 detection under the conditions evaluated. Although IF and IP applications have thus far been evaluated only for DENV1 and DENV2, its broad serotype reactivity in WB suggests that it may also be suitable for similar applications involving DENV3 and DENV4.

Collectively, these findings suggest that incorporating structural accessibility and conformational epitope prediction alongside conventional linear antigenicity analyses may improve recombinant immunogen selection, particularly when antibodies are intended to recognize native proteins across multiple experimental applications. In addition, the functional characterization presented here suggests that the resulting antibody may represent a useful reagent for future NS5-based research applications, including investigations into virus–host interactions and, following appropriate validation, the development of NS5-based diagnostic or research assays.

Because the MBP tag was retained during immunization, the contribution of anti-MBP antibodies to the polyclonal preparation cannot be excluded. However, the affinity-purified antibody preparation consistently recognized native NS5 in DENV-infected cells by WB, IF, and IP. In WB, the antibody detected NS5 from all four DENV serotypes, while IF staining showed a pattern consistent with the previously described subcellular localization of NS5. Moreover, the antibody enabled co-immunoprecipitation of NS3, a well-established NS5-interacting protein. These findings provide experimental evidence that the preparation contains functional antibodies capable of recognizing native DENV NS5. Because MBP is absent from the samples used in these assays, the observed NS5 reactivity cannot be attributed to recognition of the MBP fusion tag.

Beyond the validations presented here, the anti-NS5-RdRp antibody has already been used in studies investigating Extracellular Vesicles (EVs) released from DENV2-infected U937 macrophages and their

contribution to endothelial activation [29,30]. These studies demonstrate the ability of the antibody to detect viral components in complex biological samples and suggest its potential utility as a research reagent for investigating DENV biology. Future studies may explore whether this antibody has additional value in the development of diagnostic assays or other NS5-targeted applications.

Limitations

This study has three principal limitations. First, the antibody was generated and characterized exclusively in the rat model used in this work. Therefore, the conclusions are limited to this rat-derived polyclonal antibody and should not be extrapolated to antibodies produced in other host species without experimental validation. Future studies will be required to determine whether comparable performance can be achieved using the same immunogens in other host species.

Second, the specificity analyses were restricted to DENV NS5. Consequently, additional studies evaluating potential cross-reactivity with NS5 proteins from other medically relevant flaviviruses will be required to establish the broader specificity of this antibody.

Third, the comparative evaluation was performed using a single commercially available anti-NS5 antibody selected on the basis of its reported pan-serotype reactivity and prior validation in relevant immunodetection platforms. Although this comparator represents one of the best-characterized commercially available polyclonal anti-NS5 antibodies, future studies including additional commercial antibodies would provide a broader benchmark for evaluating the performance of the antibody developed in this study.

Conclusion

In this study, we developed and characterized a rat-derived polyclonal antibody against a conserved region of the DENV NS5 RdRp domain that recognizes NS5 from all four DENV serotypes and is compatible with multiple immunodetection applications. Compared with a commercially available antibody, it exhibited reduced nonspecific signals while maintaining efficient detection of native NS5 and enabling co-immunoprecipitation of NS3. Retrospective structural analyses further suggest that structural accessibility and conformational epitope distribution may contribute to immunogen performance, highlighting the potential value of integrating structural information into recombinant immunogen design. Collectively, these findings identify this antibody as a useful reagent for future investigations into DENV biology and provide a foundation for the development of additional NS5-based research and diagnostic applications following appropriate validation.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Grammarly and ChatGPT to improve the readability, language, and syntax of the manuscript. These tools were used solely to assist with language editing and writing refinement and were not used to generate, analyze, or interpret scientific data or to draw scientific conclusions. After using these tools, the authors carefully reviewed and edited the manuscript and take full responsibility for its content and scientific integrity.

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Conflict of Interest

The authors declare no conflicts of interest associated with this manuscript.

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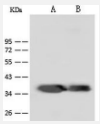
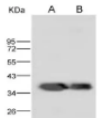
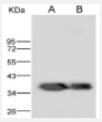
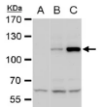
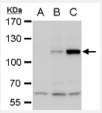
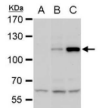
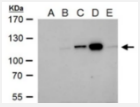
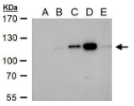
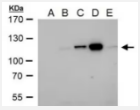
Supplementary Table S1. Commercially available polyclonal antibodies targeting dengue virus NS5 protein

Antibody reference	NS5 domain region (AA)	Antigen	Antibody–serotype reactivity (datasheet-based) serotype/Assay dilution			
			WB		IF	IP
1. PA5-112337, Thermo Scientific	M-Transferase ND	Recombinant protein	Denv2 1:20,000-1:50,000 Publications: No		Not Tested	Not tested
2. MBS2563738, MyBioSource	M-Transferase ND	Recombinant protein	Denv2 1:20,000-1:50,000 Publications: No		Not Tested	Not tested
3. 40267-T62, Sino Biological	M-Transferase ND	Recombinant protein	Denv2 1:20,000-1:50,000 Publications: No		Not Tested	Not tested
4. GTX103350, GeneTex	Polymerase 829-900	Synthetic peptide	Denv2, Denv3 1:1,000 - 1:10,000 Publications: Yes		Denv2 1:100 - 1:1,000 Publications: Yes	Not tested
5. PA5-27888, Thermo Scientific	Polymerase 829-900	Synthetic peptide	Denv2, Denv3 1:1,000 - 1:10,000 Publications: Yes		Not specified 1:100 - 1:1,000 Publications: No	Not tested
6. NBP2-42900, Novus Biologicals	Polymerase ND	Synthetic peptide	Denv2, Denv3 1:1,000-1:10,000 Publications: Yes		Denv2 1:100 - 1:1000 Publications: Yes	Not tested
7. GTX124253, GeneTex	Polymerase 647-850	Recombinant protein	Denv1, Denv2, Denv3, Denv4 1:500 - 1:3,000 Publications: Yes		Denv2, Denv3 1:100 - 1:2,000 Publications: Yes	Not tested
8. PA5-32200, Thermo Scientific	Polymerase 647-850	Recombinant protein	Denv1, Denv2, Denv3, Denv4 1:500 - 1:3,000 Publications: Yes		Not specified 1:100 - 1:2,000 Publications: No	Not tested
9. NBP2-42901, Novus Biologicals	Polymerase ND	Recombinant protein	Denv1, Denv2, Denv3, Denv4 1:500 - 1:3,000 Publications: No		Denv2, Denv3 1:100 - 1:2,000 Publications: No	Not tested

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3. https://www.sinobiological.com/antibodies/denv-ns5a-40267-t62
4. https://www.genetex.com/Product/Detail/Dengue-virus-NS5-protein-antibody/GTX103350?srsltid=AfmBOooOpu3Z3wz7DWgeZYCYagwLaqeqhwo0zWopPgyP0BkMU4r2BSKw
5. https://www.thermofisher.com/antibody/product/Dengue-Virus-Type-2-NS5-Antibody-Polyclonal/PA5-27888
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This table summarizes key characteristics of commercially available polyclonal antibodies raised against different domains of the dengue virus NS5 protein. Details include antibody reference, target domain and amino acid region, nature of the immunogen, reported serotype specificity, recommended dilutions for western blot (WB), immunofluorescence (IF), and immunoprecipitation (IP), and availability of supporting publications.

Supplementary Table S2. Commercial anti NS5 monoclonal antibodies

Antibody reference	NS5 domain region (AA)	Antigen	Antibody–serotype reactivity (datasheet-based) serotype/Assay dilution		
			WB	IF	IP
1. GeneTex, GTX629446	Polymerase 829-900	Synthetic peptide	Denv2, Denv3 1:1,000 - 1:10,000	Denv2, Denv3 1:100 - 1:2,000	Denv2 Publications: 1[Ref]
2. GeneTex, GTX629447	Polymerase 829-900	Synthetic peptide	Denv2, Denv3, Denv4 1:1,000 - 1:10,000	Denv2, Denv3 1:100 - 1:2,000	Not tested Publications: 0
3. Sigma, SAB2702241	Polymerase 829-900	Synthetic peptide	Denv2, Denv3 1:1,000 - 1:10,000	Denv2, Denv3 1:100 - 1:2,000	Not tested Publications: 0
4. Sigma, SAB2702242	Polymerase 829-900	Synthetic peptide	Denv2, Denv3 1:1,000 - 1:10,000	Denv2, Denv3 1:100 - 1:2,000	Not tested Publications: 0
5. Thermo Sci, MA5-17295	Polymerase 829-900	Synthetic peptide	Denv2, Denv3 1:1,000 - 1:10,000	Denv2, Denv3 1:100 - 1:2,000	Assay-dependent Publications: 0
6. Thermo Sci, MA5-17296	Polymerase 829-900	Synthetic peptide	Denv2, Denv3 1:1,000 - 1:10,000	Denv2, Denv3 1:100 - 1:2,000	Not tested Publications: 0
7. Abcam ab308390	ND	Synthetic peptide	Denv2, Denv3, 1:5000	Denv2, Denv3 1:2 000	Not tested

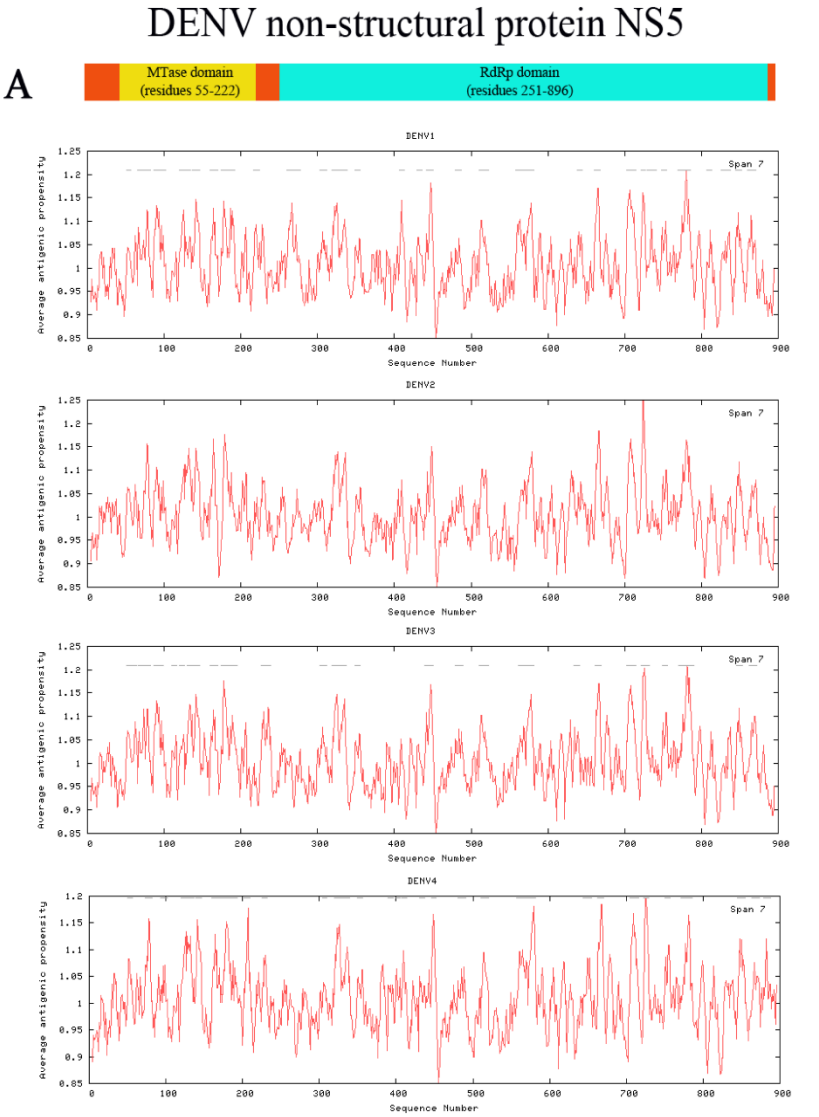
1. https://www.genetex.com/Product/Detail/Dengue-virus-NS5-protein-antibody-GT353/GTX629446?srsltid=AfmBOopCYui62oWKpicsrKGF8ysm3PedlXPpCkrOm9lqulKVksSHV01u
2. https://www.genetex.com/Product/Detail/Dengue-virus-NS5-protein-antibody-GT361/GTX629447?srsltid=AfmBOoq2FAHjneJ1J0sDI6IFgcqW3b66jek0RrVhOOSAhL3xEjTX2Lig
3. https://www.sigmaaldrich.com/CO/en/product/sigma/sab2702241?srsltid=AfmBOopvODEGEMvR1u9Zv_kwNr47ZGXm7XUGAdYJunhISoqnEPHGlPK6
4. https://www.sigmaaldrich.com/CO/en/product/sigma/sab2702242?srsltid=AfmBOorYAZ1Sxqbj1s0Luxfgdxla3mQsXduVLwuEdmNywh5OvOn5p8k8
5. https://www.thermofisher.com/antibody/product/Dengue-Virus-Type-2-NS5-Antibody-clone-GT353-Monoclonal/MA5-17295
6. https://www.thermofisher.com/antibody/product/Dengue-Virus-Type-2-NS5-Antibody-clone-GT361-Monoclonal/MA5-17296
7. https://www.abcam.com/en-us/products/primary-antibodies/dengue-virus-type-2-ns5-protein-antibody-gt361-ab308390?srsltid=AfmBOorU3NkZsTiPpuJcDNO6Lm9T8-VFdT7UdmLwf3oDo68HzQ9ewxYx

This table lists monoclonal antibodies developed against the dengue virus NS5 protein, including information on clone reference, domain specificity and amino acid region, immunogen type, reported reactivity across dengue serotypes, recommended dilutions for WB, IF, and IP applications, and the presence of supporting publications.

Supplementary Table S3. Prediction of conformational epitopes in the DENV2 NS5 protein using DiscoTope

Recombinant region	Residue ID	Residue name	Contact number	Propensity score	Discotope score
Mtase 160-232					
	174	ASN	9	0.106	-4.394
	196	ALA	13	-0.222	-6.722
	199	ARG	10	-0.094	-5.094
	200	LYS	11	0.95	-4.55
	209	PRO	16	1.495	-6.505
	211	SER	20	2.319	-7.681
	212	ARG	19	4.367	-5.133
	213	ASN	24	4.721	-7.279
	214	SER	18	4.509	-4.491
	215	THR	17	3.302	-5.198
	230	SER	11	-2.119	-7.619
RdRp 660-770					
	688	ARG	21	3.294	-7.206
	689	LYS	14	3.157	-3.843
	690	ASP	11	2.441	-3.059
	691	ILE	13	4.77	-1.73
	692	GLN	10	4.693	-0.307
	693	GLN	15	2.835	-4.665
	694	TRP	13	0.439	-6.061
	695	GLU	12	2.424	-3.576
	696	PRO	15	0.708	-6.792
	698	ARG	13	2.112	-4.388
	700	TRP	17	1.149	-7.351
	701	ASN	14	1.859	-5.141
	702	ASP	12	1.357	-4.643
	719	LYS	10	-0.52	-5.52
	720	ASP	11	-0.063	-5.563
	721	GLY	10	-1.057	-6.057
	722	ARG	15	0.585	-6.915
	747	SER	8	-3.476	-7.476

Conformational epitope prediction was performed for the recombinant regions of the methyltransferase (rMTase) and RNA-dependent RNA polymerase (rRdRp) domains of the DENV2 NS5 protein. In the rNS5-MTase region, residues 209–215 displayed positive propensity values; however, the corresponding DiscoTope scores and structural contact patterns did not support the presence of a strongly predicted surface-exposed conformational epitope. In the rNS5-RdRp region, residues 688–702 exhibited features suggestive of greater surface accessibility and conformational epitope potential, whereas residues 719–722 showed lower propensity values and more limited predicted exposure. The evaluated parameters included the number of structural contacts (lower values), propensity scores (positive values), and DiscoTope scores (values closer to zero), all of which are associated with an increased likelihood of conformational epitope recognition.



B

rNS5-MTase

```
NS5_[DENV1]   160  RTLRVLKMVEPWLRG-NQFCIKILNPYMPSVVETLEQMQRKHGGMLVRNPLSRNSTH
NS5_[DENV2]   160  RTLRVLNLVENWLNNNTQFCIKVLNPYMPSVIEKMEALQRKYGGALVRNPLSRNSTH
NS5_[DENV3]   160  RTIRVLKMVEPWLKN-NQFCIKVLNPYMPTVIEHLERLQRKHGGMLVRNPLSRNSTH
NS5_[DENV4]   160  RTLRVLKMVEPWLSSKPEFCIKVLNPYMPTVIEELEKLQRKHGGNLVRCPLSRNSTH
                   **:***::** ** .  :****:******:*:* :* :***:** *** ********

NS5_[DENV1]        EMYWVSCGTGNIVSAV  231
NS5_[DENV2]        EMYWVSNASGNIVSSV  232
NS5_[DENV3]        EMYWISNGTGNIVSSV  231
NS5_[DENV4]        EMYWVSGASGNIVSSV  232
                   ****:* .:*****:*
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rNS5-RdRP

```
NS5_[DENV1]   660  SGDDCVVKPIDDRFATALTALNDMGKVRKDIPQWEPSKGWNDWQQVPFCSHHFHQLI
NS5_[DENV2]   661  SGDDCVVKPLDDRFASALTALNDMGKIRKDIQQWEPSRGWNDWTQVPFCSHHFHELI
NS5_[DENV3]   661  SGDDCVVKPIDDRFANALLALNDMGKVRKDIPQWQPSKGWHDWQQVPFCSHHFHELI
NS5_[DENV4]   662  SGDDCVVKPLDERFGTSLLFLNDMGKVRKDIPQWEPSKGWKNWQEVPFCSHHFHKIF
                   *********:*:**..:*  ******:**** **:**:**::* :*********:::

NS5_[DENV1]        MKDGREIVVPCRNQDELVGRARVSQGAGWSLRETACLGKSYAQMWQLMYFHRR  769
NS5_[DENV2]        MKDGRVLVVPCRNQDELIGRARISQGAGWSLRETACLGKSYAQMWSLMYFHRR  770
NS5_[DENV3]        MKDGRKLVVPCRPQDELIGRARISQGAGWSLRETACLGKAYAQMWSLMYFHRR  770
NS5_[DENV4]        MKDGRSLVVPCRNQDELIGRARISQGAGWSLRETACLGKAYAQMWSLMYFHRR  771
                   ***** :***** ****:****:****************:*****.*******
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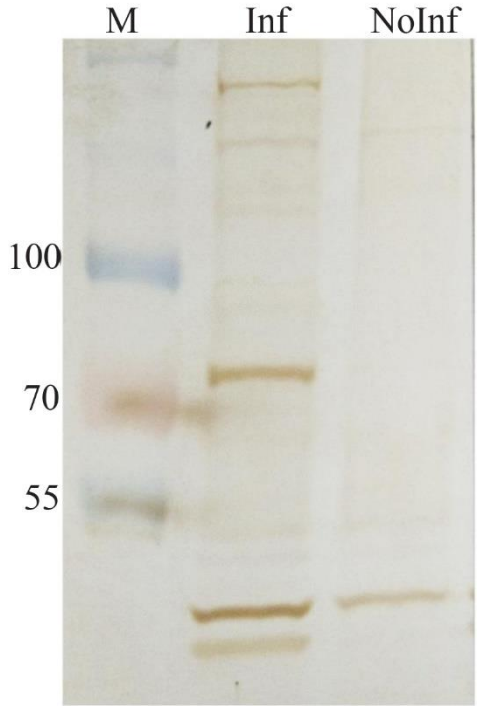
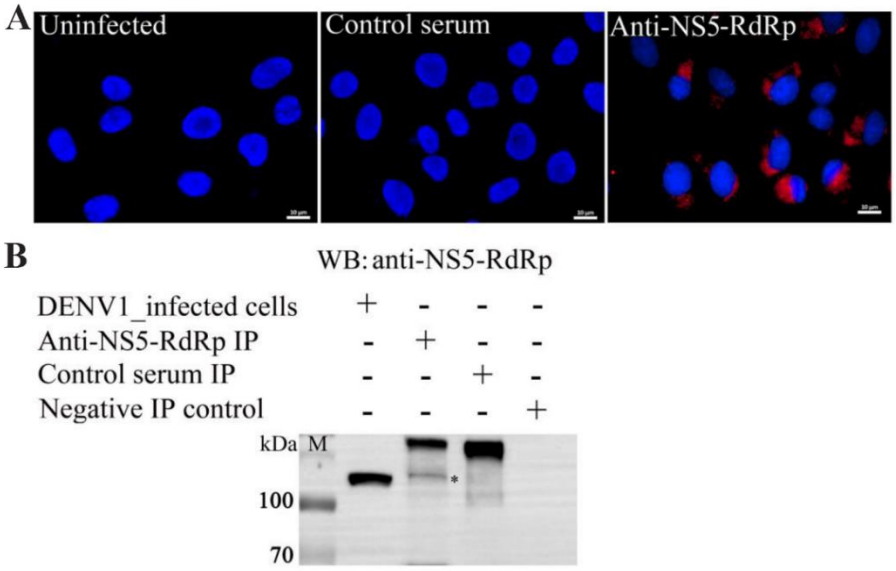
Supplementary Figure S1. Antigenicity analysis of NS5 sequences from the four DENV serotypes using the Predicted Antigenic Peptides program. A) Schematic representation of the NS5 functional domains and antigenicity profiles. Antigenicity analysis was performed to identify two independent regions for antibody generation, one within the methyltransferase (MTase) domain and the other within the RNA-dependent RNA polymerase (RdRp) domain. In the MTase domain, the highest predicted antigenic peaks were located approximately between residues 100 and 225, whereas in the RdRp domain they were observed between residues 660 and 775. Based on these predictions and primer design considerations, the regions selected for recombinant protein production and antibody generation corresponded to residues ~160–232 (MTase) and ~661–770 (RdRp). Both regions exhibited prominent antigenic peaks across all four DENV serotypes. B) Multiple sequence alignment of the selected NS5 regions from the four DENV serotypes generated with CLUSTAL O (v1.2.4) (Madeira et al., 2024). Predicted epitopes within the selected regions are highlighted by shading.



Supplementary Figure S2. Immunodetection of DENV NS5 protein in DENV2-infected BHK-21 cells using the NS5-MTase antibody. Western blot analysis of protein extracts from DENV2-infected and uninfected BHK-21 cells using crude rat serum raised against recombinant NS5-MTase (rNS5-MTase). Inf: 30 μg of DENV2-infected BHK-21 cell extract; NoInf: 30 μg of uninfected BHK-21 cell extract. M: molecular weight marker (PageRuler Plus, Fermentas). The NS5 protein was expected at approximately 105 kDa. Membranes were developed using DAB and H_2O_2. Although the serum recognized the recombinant rNS5-MTase antigen, no specific signal corresponding to endogenous NS5 was detected in infected cells despite testing multiple Western blot conditions designed to improve detection sensitivity.



Supplementary Figure S3. Immunofluorescence and immunoprecipitation assays using the anti-NS5-RdRp antibody in DENV1-infected BHK-21 cells. A) Immunofluorescence assay. Uninfected: non-infected BHK-21 cells incubated with the anti-NS5-RdRp antibody (1:500). Control serum: DENV1-infected BHK-21 cells incubated with serum from a non-immunized control rat (1:250). Anti-NS5-RdRp: DENV1-infected BHK-21 cells incubated with the anti-NS5-RdRp antibody (1:500). Scale bars: 10 μm. B) Immunoprecipitation (IP) assay followed by WB detection using the anti-NS5-RdRp antibody. M: molecular weight marker (PageRuler, Thermo Scientific). DENV1-infected cells: total protein extract from DENV1-infected BHK-21 cells. Anti-NS5-RdRp IP: IP using the anti-NS5-RdRp antibody. Control serum IP: IP using serum from a non-immunized control rat. Negative IP control: IP performed in the absence of antibody. All IP assays were performed using 1 *mg* of total protein extract from DENV1-infected BHK-21 cells. The asterisk indicates the NS5 protein band (~105 *kDa*).