Journal of Virological Methods 343 (2026) 115381

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Protocols

Integrated validation of internally controlled one-step multiplex real-time RT-PCR for dengue virus serotyping and monoplex assay for quantification

Le Thi Phuong Thuy [a], Vi Thuy Tran [a], Huynh Le Anh Huy [a], Tran Thai Hung [a], Nguyet Nguyen Minh [a], Ho Quang Chanh [a], Tran Bang Huyen [a], Nguyen Lam Vuong [a,b], Cameron P. Simmons [c], Duong Thi Hue Kien [a,*], Sophie Yacoub [a,d]

[a] Oxford University Clinical Research Unit, Wellcome Trust Major International Programme, Ho Chi Minh City, Vietnam
[b] University of Medicine and Pharmacy at Ho Chi Minh City, Ho Chi Minh City, Vietnam
[c] Monash University, Clayton 3800, Australia
[d] Pandemic Sciences Institute, University of Oxford, Oxford, UK

ARTICLE INFO

Keywords:
Dengue virus
5-PLEX multiplex real-time RT-PCR
Serotyping
Viral load quantification
Genomic surveillance
Molecular diagnostics

ABSTRACT

Dengue is a major global health challenge, yet no commercially validated molecular kits currently provide both serotyping and viral load quantification in a single integrated format. To address this gap, we developed and internally validated a one-step multiplex real-time RT-PCR assay that simultaneously detects all four dengue virus (DENV) serotypes in a single reaction (5-PLEX), together with a complementary monoplex system for quantitative viral load measurement. The 5-PLEX assay demonstrated high specificity and reliable serotyping performance during acute and early critical phases of dengue infection (day of illness ≤4). Monoplex assays for each serotype showed strong linearity, precision, and low analytical limits of detection (3–10 copies/reaction), enabling accurate quantification. Based on these findings, we propose a diagnostic framework: 5-PLEX for samples collected within the first four days of illness, the previously validated 3-PLEX assay for samples collected from day 5 of illness onward, and monoplex assays for serotype-specific viral load measurement. Internal and external process controls were incorporated to support accuracy and reproducibility within the validated framework and to facilitate future multi-laboratory evaluation. This harmonized is designed to facilitate standardized implementation across laboratories and supports improved inter-laboratory consistency in clinical diagnostics, genomic surveillance, therapeutic trials, and pathogenesis research.

1. Introduction

Dengue is a mosquito-borne viral infection caused by the dengue virus (DENV), a positive-sense single-stranded ribonucleic acid (RNA) virus belonging to the *Flavivirus* genus. There are four antigenically distinct serotypes. The clinical spectrum ranges from asymptomatic infection to severe disease, and current treatment remains supportive. Several vaccines have been developed, but none show complete or consistent effectiveness (Cracknell Daniels et al., 2025; da Silveira et al., 2019; Lee et al., 2025).

Global dengue burden has escalated rapidly. World Health Organization (WHO) reported a rise from ~500,000 cases in 2000–7.6 million in 2024 (Dengue - Global situation, 2024; World Health Organization, 2024). The disease contributes to significant Disability-Adjusted Life Years (DALYs) and economic costs, projected to reach $306 billion between 2020 and 2050 (Chen et al., 2024; Stanaway et al., 2016; Zhang et al., 2025). Factors such as climate change, vector spread, and increased mobility contribute to its global expansion (Abbasi, 2025; European Centre for Disease Prevention and Control., 2017; Khan et al., 2025; Kraemer et al., 2019; Messina et al., 2019).

Diagnostic tools for dengue can be classified into two categories: detection of host immune responses and direct viral detection. However, each of these approaches has its own important limitations. Serological assays lack sensitivity in acute phase infection (World Health Organization, 2009) and can cross-react with other flaviviruses (CDC, 2025; Hunsperger et al., 2009). Virus isolation, historically considered the gold standard for dengue diagnosis, is time consuming and resource intensive (Gyawali et al., 2017; Shu and Huang, 2004). NS1 antigen assays have high sensitivity during acute phase but lose sensitivity later and vary with immune status (Duyen et al., 2011; Tricou et al., 2011;

* Correspondence to: 764 Vo Van Kiet St, Cho Quan Ward, Ho Chi Minh City, Vietnam.
E-mail address: kiendth@oucru.org (D. Thi Hue Kien).

https://doi.org/10.1016/j.jviromet.2026.115381
Received 3 December 2025; Received in revised form 27 February 2026; Accepted 27 February 2026
Available online 2 March 2026

World Health Organization, 2009).

Real-time reverse transcription-polymerase chain reaction (RT-PCR) offers distinct advantages: detection of viral RNA during the acute phase, serotype differentiation, and quantification (World Health Organization, 2009). These benefits have led to its increasing use, particularly in pathogenesis studies and therapeutic trials (Minh Nguyen et al., 2025; Nguyen et al., 2013; Tam et al., 2012; Vuong et al., 2024, 2021; Whitehorn et al., 2016). Currently, no commercially validated diagnostic kits offer these detections in a single integrated system, and most available protocols rely on in-house assays that lack standardized validation, limiting their broader applicability. In 2011, we developed an internally validated 3-PLEX assay that combined serotyping and quantification via two separate reactions (Hue et al., 2011). While effective, it was less efficient for large-scale applications such as surveillance.

To improve workflow and harmonization, we optimized and validated a one-step multiplex real-time RT-PCR, 5-PLEX assay, that detects all four DENV serotypes with an internal control in a single reaction. The 5-PLEX assay is optimized for acute and early critical phases of dengue infection (World Health Organization, 2009), making it potentially suitable for large-scale surveillance programs. In parallel, we established a monoplex system for precise viral load quantification and retained the previously validated 3-PLEX assay as an alternative serotyping option, particularly for samples collected beyond the early critical phase. Together, this integrated framework enables efficient serotyping during clinical and epidemiological phases of infection, while supporting accurate viral load measurement for research, and pathogenesis studies.

2. Materials and methods

2.1. Primers and probes

To maximize detection across a broad spectrum of DENV strains, primer and probe alignments were performed using an extensive dataset of representative sequences. Primer/probe specificity was evaluated using Primer-BLAST (Ye et al., 2012), and primers were optimized to have similar annealing temperatures to support efficient amplification in a single-tube format.

Cross-reactivity among DENV serotypes was assessed by testing the 5-PLEX assay with 37 dengue virus strains representing all four serotypes, including both virus isolates and clinical samples (Supplementary Table S1). Analytical specificity was further evaluated using 23 RNA samples, including *Plasmodium falciparum* (n = 1, crossing point Cp = 26.43) and non-dengue viruses: Chikungunya virus (n = 1, Cp = 26.01), Zika virus (n = 1, Cp = 28.23), Japanese encephalitis virus (n = 1, Cp = 26.38), Enterovirus 71 (n = 1, Cp = 25.38), influenza A and B viruses (n = 10, mean Cp = 27.18), and SARS-CoV-2 Delta and Omicron variants (n = 8, mean Cp = 28.39). The reported Cp values correspond to amplification results obtained using the respective pathogen-specific assays. Each pathogen was tested in triplicate, and all testing procedures were identical to those used for DENV. A sample was considered positive when amplification occurred within the Cp < 35 and was reproducible in ≥ 2 of 3 replicates; absence of amplification was considered negative.

2.2. Virus isolates

DENV viruses from clinical specimens of Vietnamese patients were isolated. The strains of DENV-1, DENV-3, and DENV-4 were described in Hue et al. (2011). The DENV-2 was DENV2/Viet_Nam/2022–08–09 (GenBank: PP269906.1). These strains were used for assay optimization, validation, and as positive controls.

Equine Arteritis Virus (EAV), an enveloped, positive-sense single-stranded RNA virus, was used as an internal control and prepared following the protocol described by Hue et al. (2011). The mean Cp value for EAV was established during the initial assay setup to define quality control thresholds for routine diagnostic use. Stock EAV and external DENV positive controls for each serotype were prepared as ten-fold serial dilutions (neat to 10^{-3}). Twenty microliters of EAV was spiked into each dengue dilution prior to extraction, generating a two-dimensional dilution matrix. All samples were extracted and tested by real-time RT-PCR in triplicate across multiple routine runs over one month. Based on the resulting Cp distributions, working dilutions were selected to yield EAV Cp values within a target range of approximately 26–30. The mean Cp and coefficient of variation (CV) for EAV were calculated from these runs, and an acceptance range of ±10% of the established mean was defined and applied prospectively for routine quality control.

2.3. DNA DENV standard

DNA standard of DENV was established by plasmid clones following the protocol in Hue et al. (2011). Plasmid DNA standards were used exclusively for analytical evaluation of the quantitative monoplex assays, including assessment of linearity and analytical limits of detection.

2.4. Nucleic acid extraction and real-time one-step multiplex RT-PCR

RNA was extracted from plasma or serum samples of patients with suspected dengue infection using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche, Cat. No. 06 543 588 001) on the MagNA Pure 96 instrument. The one-step multiplex real-time RT-PCR assay was performed using the Luna® Universal Probe One-Step RT-qPCR Kit (NEB, E3006L) with 5 µL RNA in a 20 µL reaction volume, including 0.2 µM $MgCl_2$. Thermal cycling conditions consisted of three steps: reverse transcription at 57°C for 20 min, initial denaturation at 95°C for 2 min, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 20 s. The PCR system and data analysis platform were the same as described in Hue et al. (2011).

Internal (EAV) and external controls (DENV) were included in each run to monitor extraction efficiency, amplification performance, and potential contamination.

2.5. Validation of the 5-PLEX assay for serotyping and monoplex assays for quantification

Linearity, limit of detection, and precision parameters were evaluated using the monoplex format for each serotype, while specificity and positive percent agreement (PPA) were assessed using the 5-PLEX format. This validation strategy reflects the intended use of each assay, with monoplex assays applied for viral load quantification and the 5-PLEX assay applied as a qualitative serotyping tool. All validation analyses were performed using R, version 4.5.0 (R Core Team, 2025).

2.5.1. Linearity

The linearity of each serotype was assessed using eight tenfold serial dilutions of plasmid DNA, ranging from 10^7 to 10^0 copies/reaction. Each dilution was tested in quadruplicate. Linearity was evaluated by linear regression analysis, with acceptable linearity defined as $R^2 \geq 0.98$ (Marchesi et al., 2015; U.S. Food and Drug Administration, 2019).

2.5.2. Limit of detection (LOD)

The plasmid DNA was serially diluted two-fold to obtain a series of concentrations based on the linear dynamic range of each DENV serotype. Each concentration of dilution was replicated 8 times. The LOD was estimated using the Probit regression. The criteria to accept the LOD value were followed by (Hue et al., 2011). The LOD values were expressed as copies per PCR reaction, defined as the number of plasmid DNA copies added in 5 µL of standard input directly into the PCR reaction.

2.5.3. Precision

Precision was evaluated through repeatability and intermediate

precision using DENV RNA at two concentration levels. Repeatability was assessed by performing quadruplicate runs twice daily over four consecutive days, with at least two hours between runs under consistent analytical conditions (European Medicines Agency EMA, 2023). Intermediate precision was determined over the same four-day period. Repeatability and intermediate precision were analyzed using Levene's test and one-way ANOVA, respectively, following Hue et al. (2011).

2.5.4. Positive percent agreement (PPA)

We evaluated the 5-PLEX assay as a front-end qualitative serotyping tool across the course of dengue illness, from the acute phase through the recovery phase (World Health Organization, 2009), using PPA (U.S. Food and Drug Administration, 2007). Clinical samples collected across day 1–8 of illness were analyzed. The previously validated 3-PLEX assay served as an internally reference for comparative serotyping. The Cp values were derived from the quantitative monoplex. To define the operational window for reliable qualitative serotyping, a PPA threshold of $\geq$ 85% was applied to determine the appropriate use of the 5-PLEX assay.

To control for viremia, samples were stratified by Cp bands from the reference assay, and PPA was calculated within each band. Serotype-specific detection at equivalent viremia levels was compared using Fisher's exact test, with Holm–Bonferroni adjustment for multiple comparisons. Effect sizes were reported as differences in proportions (Δpp) between serotype-specific PPAs.

3. Result

3.1. Specificity of primers and probes

Primers/ probes for DENV-1, DENV-3, and DENV-4 from Hue et al. (2011) were updated, while DENV-2 primers and probes were fully redesigned to ensure coverage of all genotypes. Primers/ probes were designed and aligned against a dataset of over 4000 representative DENV sequences to ensure broad strain coverage (1310 DENV-1, 1753 DENV-2, 805 DENV-3, and 250 DENV-4), including isolates from diverse geographic regions, with emphasis on Asia and the Americas. Primer and probe sequences are detailed in Table 1.

For the specificity test using the 5-PLEX assay, each serotype-specific probe produced a signal only with its corresponding DENV serotype, with no cross-reactivity observed in RNA samples from non-dengue pathogens.

3.2. Linearity

Linearity was assessed using plasmid DNA standards, with results summarized in Table 2. DENV-1 and DENV-3 showed linearity from 10^7 to 10^0 copies/reaction, while DENV-2 and DENV-4 ranged from 10^7 to 10^1 copies/reaction (Fig. 1). All assays demonstrated robust linearity ($R^2 = 0.992–0.998$) with statistically significant regressions ($p < 0.001$). Amplification efficiencies ranged from 89.3% to 104.8%, and slopes between –3.220 and –3.600, indicating consistent target amplification across serotypes (U.S. Food and Drug Administration, 2019).

3.3. Limit of detection (LOD)-plasmid DNA

Limits of detection (LOD) were determined by Probit regression analysis, with LODs for DENV-1, DENV-2, DENV-3, and DENV-4 established at 10, 3, 7, and 8 copies/reaction, respectively, representing the lowest concentrations detected with 95% confidence. To ensure reliable quantification, all LODs fell within the assay's linear dynamic range: 10 copies/reaction for DENV-1, DENV-2, and DENV-4, and 7 copies/reaction for DENV-3.

3.4. Precision

Repeatability was assessed by testing four DENV serotypes at two concentrations, twice daily over four days. No significant variation in Cp

Table 2
Linear regression parameters for standard curves of DENV-1 to DENV-4.

	Estimate	Standard error	p-value	R^2	Linear range
DENV–1				0.998	10^7– 10^0
Intercept	38.129	0.118	< 0.001		
Slope	-3.220	0.028	< 0.001		
DENV–2				0.997	10^7– 10^1
Intercept	40.660	0.153	< 0.001		
Slope	-3.439	0.034	< 0.001		
DENV–3				0.992	10^7– 10^0
Intercept	39.064	0.238	< 0.001		
Slope	-3.465	0.057	< 0.001		
DENV–4				0.992	10^7– 10^1
Intercept	41.573	0.280	< 0.001		
Slope	-3.601	0.063	< 0.001		

Standard curves were generated for each DENV serotype using serial tenfold dilutions of plasmid DNA (10^7 to 10^0 copies/reaction). Reported parameters include the intercept, slope, standard error, p-value, coefficient of determination (R^2), and linear range are reported. A strong linear correlation was observed for all DENV serotypes.

Table 1
Primers/probes sequences for DENV serotyping and quantification.

Serotype	Primer/probe sequences (5'−3')	Position	Final conc. (μM/reaction)
DENV-1-F	ATCCATGCCCAYCAYCAATG	9865–9884	1.00
DENV-1-R	GRGTTTTRTCCTCCATCCATG	9941–9961	1.00
DENV-1-Probe	FAM-TCAGTGTGGAATAGGGTTTGGATAGAGGAA-BHQ1	9907–9936	0.14
DENV-2-F	TAYTCYATGTGYACAGGAAAG	1735–1755	1.00
DENV-2-R	ARCCRTCYCCTTCRTATTG	1813–1831	1.00
DENV-2-Probe	HEX-AAGGAAATAGCAGAAACACAACATGGAAC-BHQ1	1768–1796	0.14
DENV-3-F	TTTCTGCTCCCACCACTTTC	9591–9610	1.00
DENV-3-R	CCATCCYGCTCCTTGAGA	9691–9708	1.00
DENV-3-Probe	ROX-AAGAAAGTTGGTAGTTCCCTGCAGACCCCA-BHQ2	9633–9662	0.14
DENV-4-F	GYGTGGTGAAGCCYCTRGAT	9587–9606	1.00
DENV-4-R	AGTGARCGGCCATCCTTCAT	9744–9763	1.00
DENV-4-Probe	Cyan500-ACTTCCCTCCTCTTYTTGAACGACATGGGA-BHQ1	9490–9519	0.14
EAV-F	CATCTCTTGCTTTGCTCCTTAG	1847–1868	0.20
EAV-R	AGCCGCACCTTCACATTG	1980–1997	0.20
EAV-Probe	Cy5-CGCGCTCGCTGTCAGAACAACATTATTGCCCACAGCGCG-BHQ3	1926–1964	0.08

Primer and probe sequences for the detection of DENV serotypes and EAV, including genomic positions and final concentrations (conc.) optimized for a 20 μL PCR reaction. F: forward primer; R: reverse primer; R = A or G (purine); Y = C or T (pyrimidine). Fluorophores and quenchers: FAM (6-carboxyfluorescein), BHQ1 (Black Hole Quencher 1), HEX (Hexachlorofluorescein), ROX (carboxy-X-rhodamine), Cyan500 (Cyan 500).

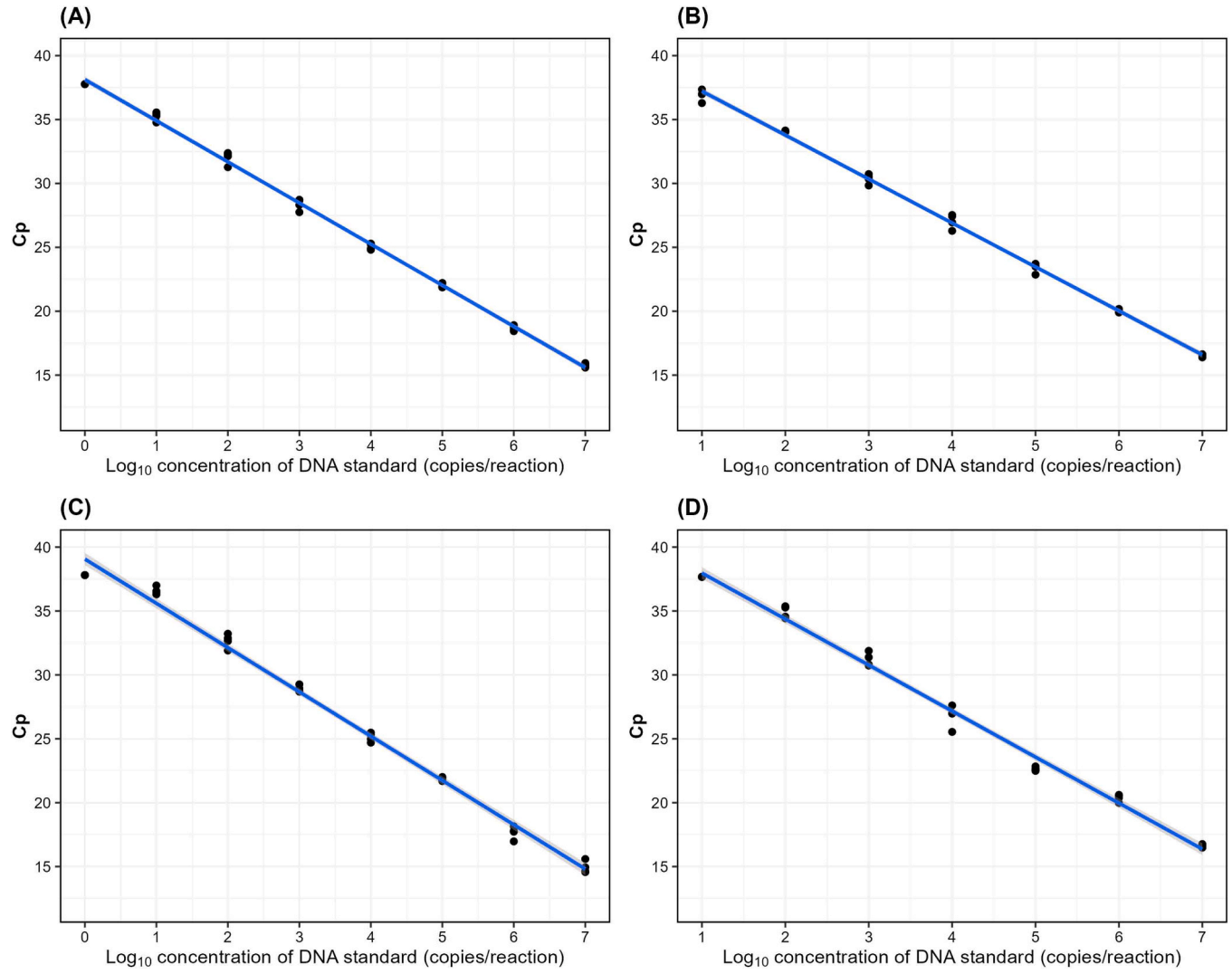

Fig. 1. Linear dynamic range of internal real-time one-step multiplex RT-PCR with eight tenfold serial dilutions of plasmid DNA. (A): DENV-1, (B): DENV-2, (C): DENV-3, (D): DENV-4.

values was observed between runs ($p > 0.05$; Table 3). Intermediate precision over the same period also showed no significant day-to-day differences by ANOVA (Table 4), confirming the assay's high reproducibility.

Table 3
Levene's test for repeatability of four DENV serotypes at medium and low RNA concentrations.

Serotype	Concentration	Cp mean	SD	p-value	CV%
DENV-1	Mid	25.86	0.37	0.43	1.44
	Low	26.97	0.17	1.00	0.61
DENV-2	Mid	22.91	0.16	0.33	0.69
	Low	23.73	0.14	0.96	0.57
DENV-3	Mid	24.80	0.28	0.77	1.12
	Low	25.74	0.27	0.13	1.03
DENV-4	Mid	24.28	0.30	0.72	1.23
	Low	25.26	0.11	0.31	0.44

Repeatability was assessed by evaluating the homogeneity of variance for each DENV serotype at medium and low RNA concentrations using Levene's test. Mean Crossing point (Cp) values, standard deviations (SD), corresponding p-values, and coefficients of variation (CV%) are presented. Non-significant p-values ($p > 0.05$) indicate no significant differences in variance, supporting consistent assay performance across replicates.

3.5. Positive percent agreement (PPA)

The operational window of qualitative serotyping performance of the 5-PLEX assay was evaluated by assessing PPA with the internally validated 3-PLEX reference assay. Clinical samples positive for DENV-3 and DENV-4 were insufficient for inclusion in the PPA analysis. Among 353 confirmed dengue-positive samples (Figs. 2), 57.5% (203/353) were DENV-1 and 42.5% (150/353) were DENV-2, reflecting the predominant circulating serotypes in Vietnam. One hundred and ten samples (110/353, 31.2%) were collected on day 4 of illness, providing a representative basis for comparison.

Overall agreement between the two assays was first evaluated, followed by stratified analyses by day of illness (DOI), viral load (as indicated by Cp values), and serotype-specific performance (Tables 5–6). The 5-PLEX assay achieved an overall PPA of 81.6% (95% CI: 77.2–85.3). Agreement was highest during early illness, declining with disease progression from 100% on DOI 2 and 92.7% on DOI 3, to 76.8% on DOI 5 and 57.1% on DOI 6. Samples from DOI 1 ($n = 3$) and DOI 8 ($n = 1$) were excluded from interpretation due to limited numbers.

To assess the influence of viremia on detection performance, samples were further stratified by Cp value bands based on the 3-PLEX reference assay (Table 5). PPA decreased with increasing Cp: 100% for Cp $\leq$ 27, 98.9% for 27 $<$Cp$\leq$ 30, 70.8% for 30 $<$Cp$\leq$ 33, and 20.8% for Cp $>$ 33.

Table 4
Intermediate precision test results for DENV-1 to DENV-4 using one-way ANOVA (F-test).

Serotype	Conc.	Df* (between/ within)	Sum squares	Mean squares	F value	p-value	CV%
DENV-1	Mid	3/28	0.17	0.06	0.28	0.84	1.68
	Low	3/28	0.10	0.03	0.42	0.74	1.02
DENV-2	Mid	3/28	3.03	1.01	1.55	0.23	3.57
	Low	3/28	1.05	0.35	0.69	0.57	2.92
DENV-3	Mid	3/28	0.37	0.12	2.50	0.08	7.22
	Low	3/28	0.26	0.09	2.16	0.12	5.90
DENV-4	Mid	3/28	8.26	2.75	2.38	0.09	4.61
	Low	3/28	5.47	1.82	1.40	0.26	4.52

Intermediate precision was evaluated for DENV-1 to DENV-4 at medium and low RNA concentrations (Conc.) using one-way ANOVA. The result of one-way ANOVA and coefficients of variation (CV%) of Cp value were reported. For all serotypes and concentrations, p-values exceeded 0.05, indicating no statistically significant differences between runs, thus confirming consistent performance under intermediate precision conditions.

* Df: Degree of freedom

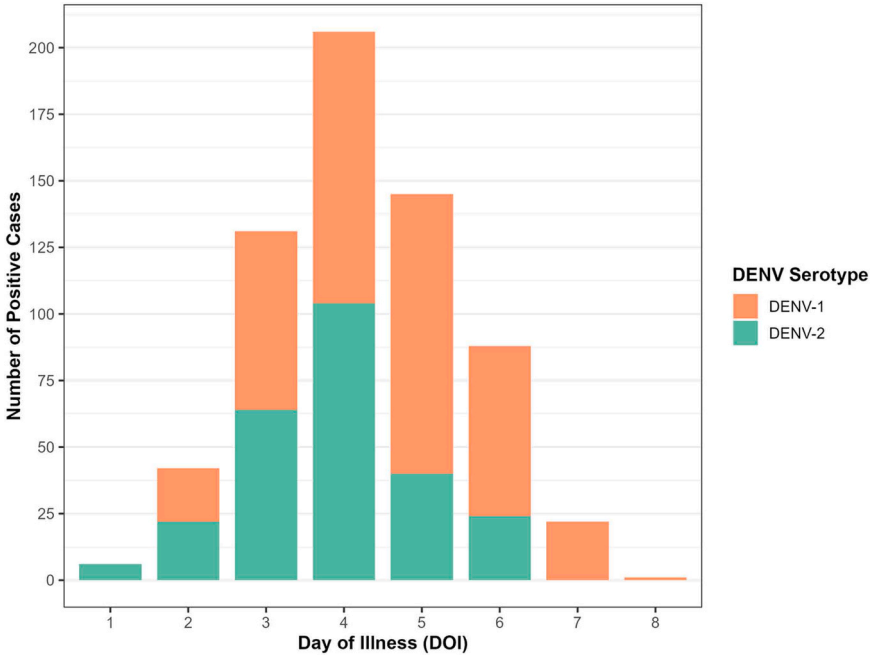

Fig. 2. Distribution of Day of Illness (DOI) among 353 confirmed dengue-positive samples by reference assay (3-PLEX), stratified by DENV serotype. Bar chart shows the number of DENV-1 and DENV-2 positive samples by DOI at time of sampling, based on results from the 3-PLEX assay. The majority of cases were detected between DOI-3 and 5.

This gradient closely paralleled the decline observed across illness days, highlighting viremia level as a major determinant of agreement (Tricou et al., 2011; Vuong et al., 2024).

We also compared serotype-specific agreement within each Cp range (Table 6). Performance for DENV-1 and DENV-2 was comparable at Cp $\leq$ 33. However, in samples with Cp $>$ 33, DENV-1 showed significantly higher agreement than DENV-2 (42.1% vs. 6.9%; p = 0.03; Δ = 35.2 % points).

These findings confirm that the 5-PLEX assay performs reliably for serotyping DENV-1 and DENV-2 up to DOI 4 (PPA= 87.27%) and Cp $\leq$ 30 (98.86%). Discrepancies beyond this range occurred in the late critical phase (DOI $\geq$ 5). The decline in PPA observed after DOI 4 reflects the natural kinetics of dengue viremia. During the later stage of the critical phase, viral loads decrease rapidly as immune-mediated viral clearance progresses. Under these low-viremia conditions, qualitative detection becomes inherently more challenging, particularly in multiplex formats where competition among primer-probe sets may reduce sensitivity compared to monoplex reactions. Therefore, the reduced PPA observed at Cp $>$ 30 does not indicate assay failure but instead reflects the biological constraints of declining viremia during the late critical phase (DOI $\geq$5) and the recovery phase of dengue infection.

Based on these results, we determined that the operational window of the 5-PLEX assay for DENV-1 and DENV-2 corresponds to samples collected at DOI $\leq$ 4, where PPA remains $\geq$ 85%. Overall, the 5-PLEX assay is well-suited as a front-line serotyping tool for samples collected during the acute and early critical phases of dengue infection.

3.6. *DENV serotyping and quantification framework*

We established a two-step framework for dengue serotyping and quantification (Fig. 3). Serotyping is performed using the 5-PLEX assay for samples collected within four days of illness (DOI $\leq$4) and the 3-PLEX assay for samples collected beyond DOI 4. This strategy is intended to reduce the risk of false-negative serotyping results by aligning assay selection with the expected kinetics of dengue viremia. Quantification is then conducted using the corresponding monoplex assay with serotype-specific primers, probes, and DNA standards. Non-target primers were replaced with nuclease-free water.

The reported quantification (copies/mL) is derived from the real-time RT-PCR quantification result (copies/reaction), adjusted based on

Table 5
Positive percent agreement (PPA) of the 5-PLEX assay against the 3-PLEX assay by day of illness (DOI), serotype, and Cp band.

	n/N	Mean of Cp value (min; max)	PPA (%)	Wilson 95% CI
Overall PPA	288/353		81.59	77.21−85.28
By DOI				
1	3/3	16.89 (13.86;18.42)	100.00	43.85−100.00
2	21/21	21.66 (16.42;30.99)	100.00	84.54−100.00
3	63/68	25.83 (16.77;34.82)	92.65	83.91−96.82
4	96/110	28.2 (18.87;34.57)	87.27	79.76−92.27
5	63/82	29.94 (20.11;34.44)	76.83	66.62−84.63
6	32/56	31.31 (24.73;34.35)	57.14	44.14−69.23
7	10/12	30.14 (26.65;35.43)	83.33	55.20−95.30
8	0/1	32.99 (32.99;35.99)	0.00	0.00−79.35
By 3PLEX Cp band	**n/N**	**PPA (%)**		**Wilson 95% CI**
≤ 27	128/128	100.00		97.09−100.00
27 <Cp≤ 30	87/88	98.86		93.84−99.80
30 <Cp ≤ 33	63/89	70.79		60.64−79.22
> 33	10/48	20.83		11.73−34.26

n = number of 5-PLEX–positive samples among 3-PLEX–positive specimens;
N = total number of 3-PLEX–positive specimens.

assay input and extraction volumes. Copy numbers below the serotype-specific LOD are reported as <LOD and are not converted to copies/mL. Sample validity was assessed based on EAV/DENV amplification results and whether the EAV Cp value fell within ±10% of the established mean. Samples with EAV/DENV results of (+/+), (+/–), or (–/+) were considered valid, whereas (–/–) samples were considered invalid and required repeat testing. EAV internal controls and DENV positive controls were acceptable with Cp values within the predefined range (26–30), while any amplification in the negative control invalidated the run.

4. Discussion

This study presents an internally validated and flexible molecular diagnostic framework for DENV serotyping and quantification, integrating both multiplex, 5-PLEX and 3-PLEX, and serotype-specific monoplex real-time RT-PCR assays. This integrated platform enables efficient serotyping within defined phases of infection while supporting accurate viral load measurement. With its structured and quality-controlled design, it provides a foundation for future harmonized implementation across clinical and research settings.

Multiplex assays often face challenges such as reduced sensitivity or specificity due to competition between primers and probes in a single reaction. Despite these potential limitations, the newly developed 5-PLEX assay demonstrated high specificity, no cross-reactivity with other pathogens, and reliable serotyping performance, particularly in acute and early critical phases of dengue infection. Meanwhile, the monoplex assays serve as dedicated components of the framework for precise viral load quantification. Together, they may support a wide range of downstream applications, including pathogenesis research, therapeutic evaluations, and public health efforts such as surveillance.

Real-time RT-PCR has become increasingly prevalent in DENV research; however, many existing protocols still lack standardized validation. Our recent large-scale patient study reinforced the clinical relevance of viremia as a prognostic marker for dengue severity (Vuong et al., 2024, 2021). Patients with high early viremia and slower clearance rates were at significantly greater risk of developing severe disease. These findings highlight the potential value of standardized viremia measurement for clinical risk stratification. Moreover, as viremia represents a key virological endpoint in both pathogenesis and therapeutic studies, the need for standardized molecular methods is particularly critical (Simmons et al., 2012), especially in multi-center trials where methodological consistency is essential. Harmonized protocols help minimize inter-site variability, ensuring that multi-site studies and treatment assessments reflect true biological differences rather than methodological inconsistencies. Therefore, validated and reproducible quantification tools are indispensable for generating reliable, comparable, and clinically meaningful findings across studies. Today, there is growing momentum toward developing predictive models of dengue severity that integrate variables such as viremia, age, gender, immune status, hematological indices, and host biomarkers (Madewell et al., 2024; Pang et al., 2016; Vuong et al., 2021). The predictive accuracy of these models critically depends on the quality and consistency of input data, particularly for quantitative parameters like viremia. This becomes even more important in geographically diverse or multi-center studies, where methodological variability can introduce inconsistencies that undermine model reliability. In this context, the internally validated monoplex quantitative assays described in this study provide a standardized analytical framework for viral load measurement. With high analytical precision, low plasmid-based limits of detection, and strong intra-laboratory reproducibility, supported by internal and external quality controls, this platform establishes a foundation for future multi-site implementation and harmonization efforts.

Beyond quantification, DENV serotyping is gaining recognition as an essential component of national dengue control programs, as it enables monitoring of circulating serotype patterns (Anh et al., 2025; Iqbal et al., 2024; Phan et al., 2022; Tariq et al., 2025). Notably, several studies have shown that shifts in serotype and genotype often coincide with large outbreaks and increased disease severity (Adams et al., 2006; Hang et al., 2010; Tran et al., 2023; Zhang et al., 2005). As genomic surveillance initiatives expand, a key challenge lies in the need for harmonized molecular methods to ensure consistent, high-quality datasets across regions and institutions. In this context, the 5-PLEX assay demonstrates

Table 6
Positive percent agreement (PPA) of the 5-PLEX versus 3-PLEX by Cp band and serotype (DENV-1, DENV-2).

Cp band	DENV-1 PPA (n/N, 95%CI)	DENV-2 PPA (n/N, 95%CI)	Δ pp	p (Fisher)	p (Holm)
≤ 27	100.00 (73/73, 95.00 −100.00)	100.00 (55/55, 93.47 −100.00)	0.00	1.00	1.00
27 <Cp≤ 30	100.00 (52/52, 93.12 −100.00)	97.22 (35/36, 85.83 −99.51)	+ 2.78	0.41	0.82
30 <Cp≤ 33	76.27 (45/59, 64.03 −85.31)	60.00 (18/30, 42.32 −75.41)	+ 16.27	0.14	0.42
> 33	42.11 (8/19, 23.14 −63.72)	6.90 (2/29, 1.91 −21.96)	+ 35.21	0.01	0.03

n = number of 5-PLEX–positive samples among 3-PLEX–positive specimens;
N = total number of 3-PLEX–positive specimens.

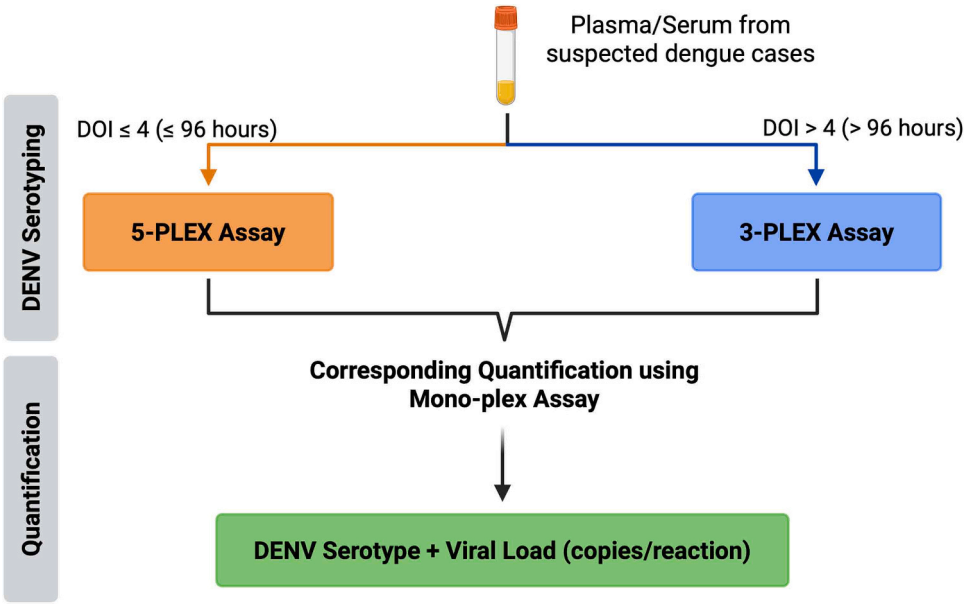

Fig. 3. Diagnostic framework for dengue serotyping and quantification using 5-PLEX, 3-PLEX, and monoplex real-time RT-PCR assays. Suspected dengue cases are tested using plasma or serum samples. Based on the day of illness (DOI), samples are directed to either the 5-PLEX assay (for DOI ≤ 4 or ≤ 96 h) or the 3-PLEX assay (for DOI >4 or >96 h) to determine the DENV serotype. Serotype-specific quantification is subsequently performed using the corresponding monoplex assay. Quality controls, including positive, internal, and negative controls, are implemented throughout all steps from RNA extraction to amplification.

potential for use in genomic surveillance and regional disease monitoring. Developed and internally validated within a structured quality-controlled framework, the platform is designed to support future harmonized implementation across multi-site studies. First, it enables reliable detection and serotyping of DENV-1 and DENV-2 during the acute and early critical phases of infection (DOI $\leq$4), when viral loads are sufficiently high to support reliable molecular detection. This is particularly relevant for surveillance activities, where variability in sample timing may influence diagnostic performance. Second, the design of primers and probes based on globally representative DENV sequence datasets, together with demonstrated analytical specificity using virus strains originating from multiple geographic regions, supports the potential regional applicability of this protocol. Third, it offers major gains in workflow efficiency and cost-effectiveness over previous assay, the 3-PLEX assay, which required separate reactions for full serotyping.

Despite these advantages, several limitations should be acknowledged. First, the PPA evaluation of the 5-PLEX was limited to DENV-1 and DENV-2; therefore, an evidence-based operational window could not be established for DENV-3 and DENV-4, restricting applicability in regions where these serotypes predominate. Second, PPA assessment was constrained during the late critical phase (DOI $\geq$5), when viral loads decline substantially. Consequently, samples collected predominantly after DOI 4 may be associated with reduced detection sensitivity of the 5-PLEX assay, which could affect serotype distribution estimates in surveillance settings. Third, although primers were designed using globally representative datasets, further laboratory validation across more diverse genotypes and geographic regions is warranted. Fourth, independent multi-site evaluations and participation in external quality assessment programs to strengthen international benchmarking of the 5-PLEX assay. Finally, implementation of these assays requires molecular infrastructure and trained personnel, which may limit uptake in resource-limited settings.

In summary, this study introduces an internally validated and scalable diagnostic platform for DENV serotyping and quantification. The 5-PLEX assay enables streamlined serotyping in a single reaction, while the monoplex assays provide quantitative viral load measurement within an integrated framework. Together, these components establish a structured and reproducible workflow that may be implemented in other laboratories and could support multi-site studies by promoting methodological consistency across research settings.

CRediT authorship contribution statement

Le Thi Phuong Thuy: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Sophie Yacoub:** Resources, Funding acquisition. **Huynh Le Anh Huy:** Investigation. **Tran Thuy Vi:** Writing – review & editing, Investigation. **Nguyet Nguyen Minh:** Resources. **Tran Thai Hung:** Validation. **Tran Bang Huyen:** Resources. **Ho Quang Chanh:** Resources. **Cameron P. Simmons:** Writing – review & editing, Resources. **Nguyen Lam Vuong:** Writing – review & editing. **Duong Thi Hue Kien:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

We thank Dr. Nguyen Thanh Thuy Nhien, Dr. Marc Choisy, and Prof. Le Van Tan for providing specimens of other pathogens used in specificity testing.

This work was supported by Wellcome [223004, https://doi.org/10.35802/223004]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2026.115381.

Data availability

Data will be made available on request.

References

Abbasi, E., 2025. The impact of climate change on travel-related vector-borne diseases: a case study on dengue virus transmission. Travel Med. Infect. Dis. 65, 102841. https://doi.org/10.1016/j.tmaid.2025.102841.

Adams, B., Holmes, E.C., Zhang, C., Mammen, M.P., Nimmannitya, S., Kalayanarooj, S., Boots, M., 2006. Cross-protective immunity can account for the alternating epidemic pattern of dengue virus serotypes circulating in Bangkok. Proc. Natl. Acad. Sci. 103, 14234–14239. https://doi.org/10.1073/pnas.0602768103.

Anh, D.D., The, N.T., My, T.N., Linh, L.T.K., Hoan, N.X., Kremsner, P.G., Toan, N.L., Song, L.H., Velavan, T.P., 2025. Epidemiology and genotype dynamics of dengue in hospitalized patients in northern vietnam between 2020 and 2022. Open Forum Infect. Dis. 12, ofae753. https://doi.org/10.1093/ofid/ofae753.

CDC, 2025. Serologic Tests for Dengue Virus [WWW Document]. Dengue. URL ⟨https://www.cdc.gov/dengue/hcp/diagnosis-testing/serologic-tests-for-dengue-virus.html⟩ (accessed 7.13.25).

Chen, S., Cao, Z., Jiao, L., Chen, W., Prettner, K., Kuhn, M., Zhao, J., Wilder-Smith, A., Bärnighausen, T., 2024. The Global Economic Burden of Dengue in 2020–2050: Estimates and Projections for 141 Countries and Territories. https://doi.org/10.2139/ssrn.4691773.

Cracknell Daniels, B., Ferguson, N.M., Dorigatti, I., 2025. Efficacy, public health impact and optimal use of the Takeda dengue vaccine. Nat. Med. 31, 2663–2672. https://doi.org/10.1038/s41591-025-03771-y.

da Silveira, L.T.C., Tura, B., Santos, M., 2019. Systematic review of dengue vaccine efficacy. BMC Infect. Dis. 19, 750. https://doi.org/10.1186/s12879-019-4369-5.

Dengue - Global situation [WWW Document], 2024. URL ⟨https://www.who.int/emergencies/disease-outbreak-news/item/2024-DON518⟩ (accessed 7.11.25).

Duyen, H.T.L., Ngoc, T.V., Ha, D.T., Hang, V.T.T., Kieu, N.T.T., Young, P.R., Farrar, J.J., Simmons, C.P., Wolbers, M., Wills, B.A., 2011. Kinetics of plasma viremia and soluble nonstructural protein 1 concentrations in dengue: differential effects according to serotype and immune status. J. Infect. Dis. 203, 1292–1300. https://doi.org/10.1093/infdis/jir014.

European Centre for Disease Prevention and Control, 2017. Vector control with a focus on Aedes aegypti and Aedes albopictus mosquitoes: literature review and analysis. Publications Office, LU.

European Medicines Agency (EMA), 2023. ICH Q2(R2) Guideline on validation of analytical procedures - Step 5.

Gyawali, N., Taylor-Robinson, A.W., Gyawali, N., Taylor-Robinson, A.W., 2017. Diagnosis of Dengue: Strengths and Limitations of Current Techniques and Prospects for Future Improvements, in: Dengue - Immunopathology and Control Strategies. IntechOpen. https://doi.org/10.5772/67680.

Hang, V.T.T., Holmes, E.C., Veasna, D., Quy, N.T., Hien, T.T., Quail, M., Churcher, C., Parkhill, J., Cardosa, J., Farrar, J., Wills, B., Lennon, N.J., Birren, B.W., Buchy, P., Henn, M.R., Simmons, C.P., 2010. Emergence of the Asian 1 genotype of dengue virus serotype 2 in viet nam: in vivo fitness advantage and lineage replacement in South-East Asia. PLoS Negl. Trop. Dis. 4, e757. https://doi.org/10.1371/journal.pntd.0000757.

Hue, K.D.T., Tuan, T.V., Thi, H.T.N., Bich, C.T.N., Anh, H.H.L., Wills, B.A., Simmons, C.P., 2011. Validation of an internally controlled one-step real-time multiplex RT-PCR assay for the detection and quantitation of dengue virus RNA in plasma. J. Virol. Methods 177, 168–173. https://doi.org/10.1016/j.jviromet.2011.08.002.

Hunsperger, E.A., Yoksan, S., Buchy, P., Nguyen, V.C., Sekaran, S.D., Enria, D.A., Pelegrino, J.L., Vázquez, S., Artsob, H., Drebot, M., Gubler, D.J., Halstead, S.B., Guzmán, M.G., Margolis, H.S., Nathanson, C.-M., Lic, N.R.R., Bessoff, K.E., Kliks, S., Peeling, R.W., 2009. Evaluation of commercially available anti–dengue virus immunoglobulin M tests. Emerg. Infect. Dis. 15, 436–439. https://doi.org/10.3201/eid1503.080923.

Iqbal, N.T., Ahmed, K., Khalid, A., Imtiaz, K., Ali, Q.M., Munir, T., Mahmood, S.F., Khan, U., Afzal, B., Qamar, F., Waggoner, J.J., Fenelon, H., McOwen, H., Khan, E., Rabinowitz, P., Van Voorhis, W.C., 2024. Clin. Charact. serotype Assoc. Dengue Dengue Illn. Pak. https://doi.org/10.1101/2024.10.02.24314748.

Khan, M., Abdalgader, T., Pedersen, M., Zhang, L., 2025. Interactive effects of climate change and human mobility on dengue transmission. Ecol. Model. 499, 110924. https://doi.org/10.1016/j.ecolmodel.2024.110924.

Kraemer, M.U.G., Reiner, R.C., Brady, O.J., Messina, J.P., Gilbert, M., Pigott, D.M., Yi, D., Johnson, K., Earl, L., Marczak, L.B., Shirude, S., Davis Weaver, N., Bisanzio, D., Perkins, T.A., Lai, S., Lu, X., Jones, P., Coelho, G.E., Carvalho, R.G., Van Bortel, W., Marsboom, C., Hendrickx, G., Schaffner, F., Moore, C.G., Nax, H.H., Bengtsson, L., Wetter, E., Tatem, A.J., Brownstein, J.S., Smith, D.L., Lambrechts, L., Cauchemez, S., Linard, C., Faria, N.R., Pybus, O.G., Scott, T.W., Liu, Q., Yu, H., Wint, G.R.W., Hay, S. I., Golding, N., 2019. Past and future spread of the arbovirus vectors Aedes aegypti and Aedes albopictus. Nat. Microbiol. 4, 854–863. https://doi.org/10.1038/s41564-019-0376-y.

Lee, M.F., Long, C.M., Poh, C.L., 2025. Current status of the development of dengue vaccines. Vaccin. X 22, 100604. https://doi.org/10.1016/j.jvacx.2024.100604.

Madewell, Z.J., Rodriguez, D.M., Thayer, M.B., Rivera-Amill, V., Paz-Bailey, G., Adams, L.E., Wong, J.M., 2024. Machine learning for predicting severe dengue, Puerto Rico. https://doi.org/10.1101/2024.11.15.24317377.

Marchesi, U., Mazzara, M., Broll, H., Giacomo, M.D., Grohmann, L., Herau, V., Holst-Jensen, A., Hougs, L., Hübert, P., Laurensse, E., Morisset, D., Papazova, N., Pietsch, K., Pla, M., Sandberg, M., Savini, C., Hans-Ulrich Waiblinger, Woll, K., 2015. European Network of GMO Laboratories (ENGL) 2015 Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing. https://doi.org/10.13140/RG.2.1.2060.5608.

Messina, J.P., Brady, O.J., Golding, N., Kraemer, M.U.G., Wint, G.R.W., Ray, S.E., Pigott, D.M., Shearer, F.M., Johnson, K., Earl, L., Marczak, L.B., Shirude, S., Davis Weaver, N., Gilbert, M., Velayudhan, R., Jones, P., Jaenisch, T., Scott, T.W., Reiner, R.C., Hay, S.I., 2019. The current and future global distribution and population at risk of dengue. Nat. Microbiol. 4, 1508–1515. https://doi.org/10.1038/s41564-019-0476-8.

Minh Nguyen, N., Thi Hoai Tam, D., Quang Chanh, H., Trung Trieu, H., Thi Xuan Chau, N., Thanh Van, N., Thanh Phong, N., Thi Tam, C., Thi Hong Lan, N., Thi Hue Tai, L., Thi Cam Huong, N., Thi Ngoc Thuan, L., Van Hao, N., Tu Qui, P., Thi Dong Vien, T., Thi Hai Men, P., Thi Thuy Hoa, H., Vinh Tho, P., Thi Le Duyen, H., Tran Thuy, V., Dang Trong, T., Tan Thanh Kieu, N., McBride, A., Kestelyn, E., Geskus, R. B., Lam Vuong, N., Yacoub, S., 2025. Safety and tolerability of metformin in overweight and obese patients with dengue: An open-label clinical trial (MeDO). PLoS Negl. Trop. Dis. 19, e0013281. https://doi.org/10.1371/journal.pntd.0013281.

Nguyen, N.M., Tran, C.N.B., Phung, L.K., Duong, K.T.H., Huynh, H. le A., Farrar, J., Nguyen, Q.T.H., Tran, H.T., Nguyen, C.V.V., Merson, L., Hoang, L.T., Hibberd, M.L., Aw, P.P.K., Wilm, A., Nagarajan, N., Nguyen, D.T., Pham, M.P., Nguyen, T.T., Javanbakht, H., Klumpp, K., Hammond, J., Petric, R., Wolbers, M., Nguyen, C.T., Simmons, C.P., 2013. A randomized, double-blind placebo controlled trial of balapiravir, a polymerase inhibitor, in adult dengue patients. J. Infect. Dis. 207, 1442–1450. https://doi.org/10.1093/infdis/jis470.

Pang, J., Lindblom, A., Tolfvenstam, T., Thein, T.-L., Naim, A.N.M., Ling, L., Chow, A., Chen, M.I.-C., Ooi, E.E., Leo, Y.S., Hibberd, M.L., 2016. Discovery and validation of prognostic biomarker models to guide triage among adult dengue patients at early infection. PLOS ONE 11, e0155993. https://doi.org/10.1371/journal.pone.0155993.

Phan, D.Q., Nguyen, L.D.N., Pham, S.T., Nguyen, T., Pham, P.T.T., Nguyen, S.T.H., Pham, D.T., Pham, H.T., Tran, D.K., Le, S.H., Pham, T.T., Nguyen, K.C.D., Dipalma, G., Inchingolo, A.D., Piscitelli, P., Miani, A., Salvatore, S., Cantore, S., Aityan, S.K., Ballini, A., Inchingolo, F., Gargiulo Isacco, C., Pham, V.H., 2022. The Distribution of Dengue Virus Serotype in Quang Nam Province (Vietnam) during the Outbreak in 2018. Int. J. Environ. Res. Public. Health 19, 1285. https://doi.org/10.3390/ijerph19031285.

R Core Team, 2025. R: A Language and Environment for Statistical Computing.

Shu, P.-Y., Huang, J.-H., 2004. Current advances in dengue diagnosis. Clin. Diagn. Lab. Immunol. 11, 642–650. https://doi.org/10.1128/CDLI.11.4.642-650.2004.

Simmons, C.P., Wolbers, M., Nguyen, M.N., Whitehorn, J., Shi, P.Y., Young, P., Petric, R., Nguyen, V.V.C., Farrar, J., Wills, B., 2012. Therapeutics for dengue: recommendations for design and conduct of early-phase clinical trials. PLoS Negl. Trop. Dis. 6, e1752. https://doi.org/10.1371/journal.pntd.0001752.

Stanaway, J.D., Shepard, D.S., Undurraga, E.A., Halasa, Y.A., Coffeng, L.E., Brady, O.J., Hay, S.I., Bedi, N., Bensenor, I.M., Castañeda-Orjuela, C.A., Chuang, T.-W., Gibney, K.B., Memish, Z.A., Rafay, A., Ukwaja, K.N., Yonemoto, N., Murray, C.J.L., 2016. The global burden of dengue: an analysis from the Global Burden of Disease Study 2013. Lancet Infect. Dis. 16, 712–723. https://doi.org/10.1016/s1473-3099(16)00026-8.

Tam, D.T.H., Ngoc, T.V., Tien, N.T.H., Kieu, N.T.T., Thuy, T.T.T., Thanh, L.T.C., Tam, C. T., Truong, N.T., Dung, N.T., Qui, P.T., Hien, T.T., Farrar, J.J., Simmons, C.P., Wolbers, M., Wills, B.A., 2012. Effects of short-course oral corticosteroid therapy in early dengue infection in vietnamese patients: a randomized, placebo-controlled trial. Clin. Infect. Dis. 55, 1216–1224. https://doi.org/10.1093/cid/cis655.

Tariq, F., Irfan, M., Farooq, S., Iqbal, H., Atia-tul-Wahab, Khan, I.A., Iftner, T., Choudhary, M.I., 2025. Dynamics and genetic variation of dengue virus serotypes circulating during the 2022 outbreak in Karachi. Sci. Rep. 15, 22703. https://doi.org/10.1038/s41598-025-07606-1.

Tran, V.T., Inward, R.P.D., Gutierrez, B., Nguyen, N.M., Nguyen, P.T., Rajendiran, I., Cao, T.T., Duong, K.T.H., Kraemer, M.U.G., Yacoub, S., 2023. Reemergence of cosmopolitan genotype dengue virus serotype 2, Southern Vietnam. Emerg. Infect. Dis. 29, 2180–2182. https://doi.org/10.3201/eid2910.230529.

Tricou, V., Minh, N.N., Farrar, J., Tran, H.T., Simmons, C.P., 2011. Kinetics of viremia and NS1 antigenemia are shaped by immune status and virus serotype in adults with dengue. PLoS Negl. Trop. Dis. 5, e1309. https://doi.org/10.1371/journal.pntd.0001309.

U.S. Food and Drug Administration, 2019. Guidelines for the Validation of Analytical Methods Using Nucleic Acid Sequenced-Based Technologies.

U.S. Food and Drug Administration, 2007. Statistical Guidance on Reporting Results from Studies Evaluating Diagnostic Tests - Guidance for Industry and FDA Staff | FDA (No. FDA-2020-D-0957). Center for Devices and Radiological Health.

Vuong, N.L., Quyen, N.T.H., Tien, N.T.H., Duong Thi Hue, K., Duyen, H.T.L., Lam, P.K., Tam, D.T.H., Van Ngoc, T., Jaenisch, T., Simmons, C.P., Yacoub, S., Wills, B.A., Geskus, R., 2024. Dengue viremia kinetics and effects on platelet count and clinical outcomes: an analysis of 2340 patients from Vietnam. eLife 13, RP92606. https://doi.org/10.7554/eLife.92606.

Vuong, N.L., Quyen, N.T.H., Tien, N.T.H., Tuan, N.M., Kien, D.T.H., Lam, P.K., Tam, D.T. H., Van Ngoc, T., Yacoub, S., Jaenisch, T., Geskus, R.B., Simmons, C.P., Wills, B.A., 2021. Higher Plasma Viremia in the Febrile Phase Is Associated With Adverse Dengue Outcomes Irrespective of Infecting Serotype or Host Immune Status: An Analysis of 5642 Vietnamese Cases. Clin. Infect. Dis. 72, e1074–e1083. https://doi.org/10.1093/cid/ciaa1840.

Whitehorn, J., Nguyen, C.V.V., Khanh, L.P., Kien, D.T.H., Quyen, N.T.H., Tran, N.T.T., Hang, N.T., Truong, N.T., Hue Tai, L.T., Cam Huong, N.T., Nhon, V.T., Van Tram, T.,

Farrar, J., Wolbers, M., Simmons, C.P., Wills, B., 2016. Lovastatin for the Treatment of Adult Patients With Dengue: A Randomized, Double-Blind, Placebo-Controlled Trial. In: Clin. Infect. Dis, 62. Off. Publ. Infect. Dis. Soc. Am, pp. 468–476. https://doi.org/10.1093/cid/civ949.

World Health Organization, 2024. Dengue and severe dengue [WWW Document]. URL ⟨https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue⟩ (accessed 5.9.25).

World Health Organization, 2009. Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control: New Edition, WHO Guidelines Approved by the Guidelines Review Committee. World Health Organization, Geneva.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T.L., 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinforma. 13, 134. https://doi.org/10.1186/1471-2105-13-134.

Zhang, C., Mammen, M.P., Chinnawirotpisan, P., Klungthong, C., Rodpradit, P., Monkongdee, P., Nimmannitya, S., Kalayanarooj, S., Holmes, E.C., 2005. Clade replacements in dengue virus serotypes 1 and 3 are associated with changing serotype prevalence. J. Virol. 79, 15123–15130. https://doi.org/10.1128/JVI.79.24.15123-15130.2005.

Zhang, W.-X., Zhao, T.-Y., Wang, C.-C., He, Y., Lu, H.-Z., Zhang, H.-T., Wang, L.-M., Zhang, M., Li, C.-X., Deng, S.-Q., 2025. Assessing the global dengue burden: incidence, mortality, and disability trends over three decades. PLoS Negl. Trop. Dis. 19, e0012932. https://doi.org/10.1371/journal.pntd.0012932.