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A combined molecular strategy for robust detection and rapid clade classification of sylvatic dengue virus 2 (DENV-2/GVI)

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Research Article

Keywords: Dengue virus 2, sylvatic DENV-2, DENV-2/GVI, RT-qPCR, Multiplex, Molecular surveillance, One health

Posted Date: November 17th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11610819/v1

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Additional Declarations: No competing interests reported.

Abstract

Background.

The sylvatic genotype of dengue virus 2 (DENV-2/GVI) circulates between non-human primates and forest mosquitoes in West Africa and Asia, but spillover infections to humans have been documented in Senegal and Malaysia. These strains are frequently reported as "undetermined serotype" by commercial assays, and a previously validated RT-qPCR covers only the African clade, leaving the divergent Malaysian clade undetected.

Methods.

Using a curated set of 25 complete or near-complete DENV-2/GVI genomes, we identified conserved yet clade-discriminatory regions in the E (African clade) and NS5 (Malaysian clade) genes and designed oligonucleotides in two formats: a dual-target format for grouped detection (all probes FAM-labelled) and a multiplex format for simultaneous differential detection and classification (African probe Cy5, Malaysian probe FAM). Six in vitro-transcribed RNA (ivRNA) standards spanning both clades and a wide temporal range were used for analytical validation.

Results.

Both formats amplified all six ivRNA standards without cross-amplification, with paired dual–multiplex Ct differences below ± 1.41. For the most recent circulating standards (AF_2020 and MY_2024), analytical sensitivity was comparable between formats (LoD95 ≈ 211.7 and 8.3 copies/reaction, respectively). The MY_2008 standard showed a format-dependent difference (LoD 6.9 vs 64.2 copies/reaction) that remains to be resolved. No cross-reactivity was observed against urban DENV-1–4. Clinical validation using African samples shows comparable results in both formats.

Conclusions.

This combined strategy extends molecular detection of sylvatic DENV-2 to both the African and Malaysian clades in a single reaction, enabling rapid detection and lineage classification without systematic genome sequencing. The present work is mainly analytical; despite validation on African clade natural isolates and clinical samples expansion to Malaysian Clade counterpart samples is encouraged.

Background

Sylvatic lineages of DENV-2 (DENV-2/GVI) circulate between non-human primates and forest mosquitoes in West Africa and Asia [1]. Human infections by DENV-2/GVI have recently been

documented in Senegal (Kédougou 2020, Kolda 2021) [2, 3] and, more recently, in Malaysia [1]. Several studies have shown that DENV-2/GVI-positive samples can remain "undetermined serotype" with commercial assays, most likely due to mismatches between primers or probes and sylvatic-lineage sequences [4]. This limitation can mask the circulation of divergent variants in surveillance systems where routine sequencing of strains is not systematic [5].

To facilitate molecular detection and identification, a RT-qPCR system specific to the African clade was validated in 2023 [6]. However, the non-typeable Malaysian cases belong to a distinct clade [1], that this assay does not cover. In this study we designed and validated a combined strategy covering both the African and Asian lineages of DENV-2/GVI. Our objective was to compare a dual-target format, intended for grouped detection, with a multiplex format, intended for the detection and classification of both clades in a single reaction.

Methods

Dual and Multiplex Strategies Overview

To capture the full known diversity of sylvatic DENV-2 while still resolving its geographical origin, we targeted two conserved but clade-discriminatory genomic regions: the envelope (E) gene for the African clade and the NS5 gene for the Malaysian clade (Fig. 1). Two assay formats were built around these targets. In the first, a singleplex dual-target format, each clade is detected in a separate reaction with probes carrying the same fluorophore (FAM); this provides sensitive, grouped detection of DENV-2/GVI, with lineage inferred from which target amplifies. In the second, a multiplex format, both targets are co-amplified in a single reaction using spectrally distinct probes (FAM and Cy5), allowing simultaneous detection and direct discrimination of the African and Malaysian clades in one tube. The two formats share the same primer sets, so they can be deployed interchangeably depending on whether a laboratory prioritizes simple screening or one-step lineage classification.

Genome Sequences Retrieval, Dataset Construction and Phylogenetic Tree for Clade Validation

Complete and near-complete DENV-2/GVI genomes were retrieved from GISAID [7] using the filters: length > 10 kb and ambiguous (N) base content < 10%. The primary dataset was aligned with MAFFT [8] and visually inspected in AliView [9]. Sequence headers were standardized to retain accession, putative clade and collection date. The final dataset (n = 25) was used to infer a maximum-likelihood (ML) tree (Fig. 2) that guided the selection of sequences spanning the full DENV-2/GVI diversity and a wide time frame within each clade for ivRNA generation.

Oligos Design in Dual and Multiplex Format

From the curated DENV-2/GVI dataset (n = 25) we identified two regions that were conserved within, yet discriminatory between, the two clades: a 162-bp region in the E gene for the African clade (AF) and a 141-bp region in the NS5 gene for the Malaysian clade (MY). In the dual-target format all probes were labelled with CY5; in the multiplex format the AF probe was labelled with Cy5 and the MY probe with FAM. Designed oligonucleotides were checked by BLAST to assess specificity and to rule out off-target binding to closely related viruses.

Table 1
RT-qPCR oligonucleotide sequences designed during this study for detection and differentiation of DENV-2/GVI strains.

Oligos Name	Sequence 5'-3'	Dye - Quencher	Genome Position
SylvaAF_FW	ACAGRTTATGGCACGATCACGATG		1366–1389[#]
SylvaAF_RV	TCCATCTGCAGCAGGACCAT		1426–1445[#]
SylvaAF_P	TGCTCCCCACGAACAGGTCTCGATTTYAA	Cy5-BHQ2	1393–1421[#]
SylvaMY_FW	CATGTGTGTACAAYATGATGGGAAAGAGAGAGA		8915–8947
SylvaMY_RV	GGTCTTCATTTAAGAAACCTAGRGCYTC		9028–9055
SylvaMY_P_Mtx	ARGCAAAAGGYAGCAGAGCCATATGGTA	Fam-BHQ1	8969–8996
SylvaMY_P_Dual	ARgCAAAAggCAgCAgAgCCATATggTA	Cy5-BHQ2	8969–8996

[#] Position according in reference to the isolate EEB-17, Genome GenBank accession number JF260983

[$] Position according in reference to the isolate P8-1407, Genome GenBank accession number EF105379

Design and Synthesis of ivRNA

In vitro-transcribed RNAs (ivRNAs) MY_2024, MY_2008, MY_1970, AF_2020, AF_2000 and AF_1966 spanning the binding sites of the designed RT-qPCR assays were designed, saved in FASTA format and ordered from TIB Molbiol (Berlin, Germany). Transcribed RNA was resolved at a concentration of 10^{10} RNA molecules/µl according to the manufacturer's instructions.

Analytical Sensitivity, Specificity tests and Clinical Validation

The sensitivity of the newly designed DENV-2 RT-qPCR assay was determined using 10-fold dilutions of synthetized ivRNA (10^5 – 10^0 copies per reaction) in five replicates as previously described (Dieng et al.,

2023b). The specificity was evaluated by using extracted RNA from archived sylvatic DENV-2/GVI isolated in cell culture (using vero E6 cells), and DENV 1–3 positive (Table S1).

For clinical validation RNA from human sera collected from a patient infected by DENV-2/GVI in the Kolda region in 2021 was used in addition to raw sera spiked at a ratio of 1/50 with DENV-2/GVI isolates from the Virology department biobank (Table S2).

Before the sensitivity tests the presence of RNA in all DENV samples were tested by panDENV RT-PCR using the previously described protocol [6]. All RT-qPCR reactions were performed using Lightmix 1Step RT-qPCR enzyme as previously described (*ref*) using a CFX96 thermocycler and the following temperature profile: 50° – 15mn followed by Denaturation at 95° / 1 minute and 40 cycles of 95°C / 15 seconds and 60°C / 30 seconds. Fluoresence was read and recorded by activating FAM and CY5 channel.

Results

Selection and Validation of ivRNA Design

From the topology of the phylogenetic tree and taking in account the two clades (AF & MY) and the wider temporal distribution six ivRNA standard molecules (Table 1) spanning clade specific oligo binding sites were generated (Fig. 2).

No cross detection was observed with any of the ivRNA tested in both dual and multiplexed format at 10^8 copies/µl reaction. The dual singleplex and multiplex format yielded delta Ct values within the natural variation of real time PCR (± 1.43 CT) [10] (Table 1). The lowest CT values were obtained with the ivRNA for AF2021.

Table 1
Validation of ivRNA in dual and multiplex format

ivRNA Standard	Clade	Dual Target (Cy5)	Multiplex (Fam)	Multiplex (Cy5)	Delta CT
MY_2024	Malaysian	17.5	16.81	NA	0.69
MY_2008	Malaysian	17.39	16.74	NA	0.65
MY_1970	Malaysian	16.84	17.74	NA	0.9
AF_2021	African	16.51	NA	16.58	0.07
AF_2000	African	16.96	NA	18.27	1.31
AF_1966	African	16.56	NA	16.47	0.09

NA = No Amplification

Analytical Sensitivity in Dual and Multiplex Format

Three of the six ivRNAs, corresponding to the most recent circulating viral strains in both clades (AF2021, MY2008, MY2024) were used for sensitivity testing. For AF2021 was selected for the African clade since it shares 99.96% nucleotide homology with the previously detected DENV-2/GVI AF detected in 2009 in Spain from tourist returning to Guinee Bissau [11]. Both Dual target and multiplex formats detected AF2021 and MY2024 with comparable performance. For the AF2021 standard the LoD95 was 211.71 copies/reaction in both dual target and multiplex format; for MY2024 it was 8.33 copies/reactions exhibiting a higher analytical sensitivity. For MY2008 the lowest LoD95 was obtained in singleplex PCR with 6.91 copies/reactions while it was a log less (64.21 copies/reactions) in the multiplex format. (Fig. 3).

Specificity

The newly designed dual/multiplex DENV-2/GVI RT-qPCR primers did not detect any other urban DENV1–3 viruses nor any other DENV serotypes/genotypes were amplified (Table S1) indicating a high specificity of the assays.

Clinical validation

The newly designed assays were able to detect DENV-2/GVI RNA values in dual and multiplex format with Cts of 29.86 and 30.10 respectively from the sample collected from a patient in Kolda in 2021 originally detected with a Ct value of 30.23 by the panDENV assay (Table S2).

All spiked serum sample with DENV-2/GVI isolates with a panDENV Ct range from 20.92–36.09 were detected in both dual and multiplex format (Fig. 4 Pane A). Comparable Ct values were also observed in spiked sera samples tested with both formats (Fig. 4 Pane B).

Discussion

Recent emergences of DENV-2/GVI were described both in Senegal and in Malaysia [1–3]. For both studies the entry point was the simple target failure of widely used commercial RT-qPCR assays for DENV molecular serotyping [1]. These two independent emergences in two different geographical areas implies that sylvatic dengue infection is to be reckoned with and has to be included in routine testing [5]. In their paper Suppiah and Colleagues pointed out the critical need of up-to-date diagnostic tool for reliable detection and of identification of DENV-2/GVI [1]. In a previous study our team developed a sensitive RT-qPCR and for rapid detection and differentiation, and tilling PCR oligos for genome sequencing of DENV-2/GVI [6]. The limitation of this assay was that it was restricted to the African Clade of the virus. In anticipation of sylvatic Dengue spreading geographically as all previous variants have done, we have now extended and validated a combined strategy for rapid detection and differentiation of DENV-2/GVI belonging to both African and Malaysian clades.

To test the analytical sensitivity across the variability of the clades we tested three representative ivRNA targets. For targets AF2021 and MY2024, the use of a second fluorophore and multiplexed reading did not impact on analytical sensitivity. Multiplexing therefore added a classification capability without any apparent loss of performance for the targets represented by these two standards. The result difference between multiplex and singleplex format for target MY2008 was about one order of magnitude. As the primers for the two targets are identical, amplification efficiency cannot be the issue. Competition between probes, fluorophore–quencher interactions or digestability of the probes might be responsible. It demonstates that in cases of highly variant viruses testing representative variant targets can help to obtain a more realistic characterization of the performance of the real time assays. However, since the difference affects only the last log steps of the analytical sensitivity the issue can be neglected for now as the general Ct of DENV-2/GVI in human cases recorded so far is CT 30.23 which corresponds to 1041.59 copies/ reaction and is x-logs above the lowest sensitivity value observed

The multiplex format could be incorporated into a surveillance strategy, to identify DENV-2/GVI cases and direct the material to confirmation by sequencing. A limitation of the study is that we did not have access to samples infected with DENV-2/GVI belonging to the Malaysian clade. Despite the fact that the in-silico analysis of the MY clade oligos exhibited no mismatches in the oligo binding site, and the corresponding ivRNA being amplified successfully we hope to assess the clinical performance of our assay in the future.

Conclusion

The study tried to fix the coverage gap of our previously published DENV-2/GVI assay specific to the African clade of the virus created by the description of variant DENV-2/GVI from Malaysia. Our proposed approach allows the laboratory working on dengue to be able to detect and discriminate both DENV-2/GVI viruses clade using RT-qPCR without the need to perform systematic genome sequencing. Additionally, the assay provides comparable performance as dual singleplex or multiplex assays.

Dengue is the most widespread arboviral infection in the world. While strategies are targeting the strains belonging to the prevalent urban cycle sylvatic variant detection of DENV-2/GVI is underdeveloped in spite of their evident circulation in West-Africa and in South-East Asia. For reliable surveillance a one health surveillance approach is urgently needed and our newly designed tool may help to strengthen low cost sylvatic DENV-2/GVI surveillance. Finally, since sylvatic strains are mainly maintained in reservoirs in nature their ongoing viral evolution inevitably leads to failure of developed molecular tools. This calls for monitoring of the genetic diversity of emerging variants and continuous updates for molecular detection tools.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests

Funding

None.

Author Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Idrissa DIENG, Agathe Shella Efire, Mignane Ndiaye and Ndeye Aminata Dia. The first draft of the manuscript was written by Idrissa DIENG, Ndeye Aminata Dia, Manfred Weidmann, Oumar Faye and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Figures

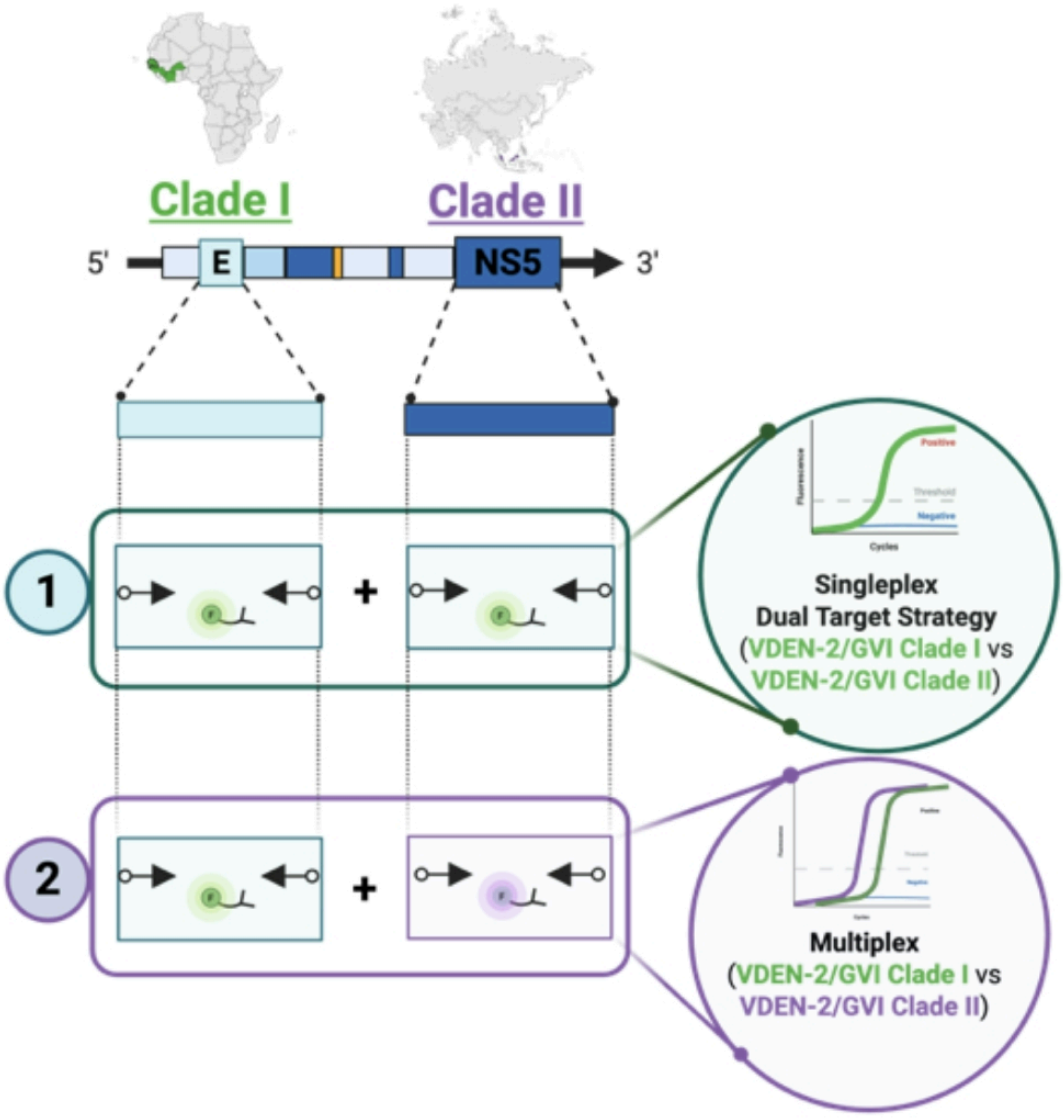


Figure 1

Combined strategy for detection and lineage classification of sylvatic DENV-2 (DENV-2/GVI). Two conserved, clade-discriminatory regions were selected from the DENV-2/GVI genome: a region in the E gene for the African clade (Clade I) and a region in the NS5 gene for the Malaysian clade (Clade II); the approximate geographical distribution of each clade is shown above. (1) Singleplex dual-target format: each clade-specific assay is run in a separate reaction with FAM-labelled probes, giving grouped (positive/negative) detection of DENV-2/GVI. (2) Multiplex format: both clade-specific assays are combined in a single reaction using two spectrally distinct fluorophores (FAM and Cy5), enabling

simultaneous detection and discrimination of the two clades. Representative amplification curves are shown for each format

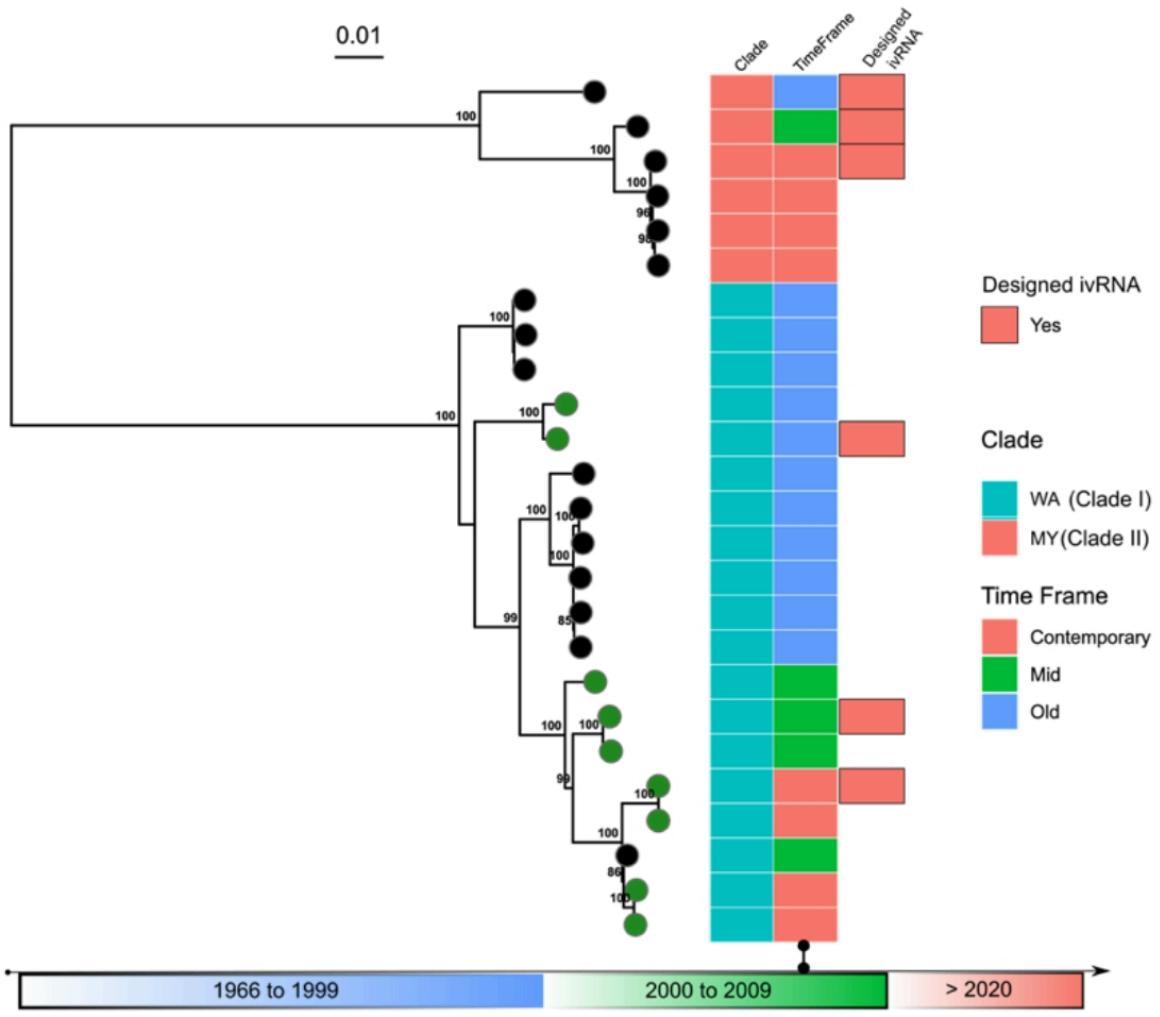


Figure 2

Maximum Likelihood generated with IQ-TREE with GTR model and 1000 replicates for robustness. The tree was generated using retrieved 25 DENV-2/GVI genomes from GISAID. Senegalese strains are colored in green. The ML tree was annotated using Clade, timeframe and those selected for the ivRNA synthesis. The third heat map column indicates the six sequences selected for the design of the ivRNA for each clade specific oligoset.

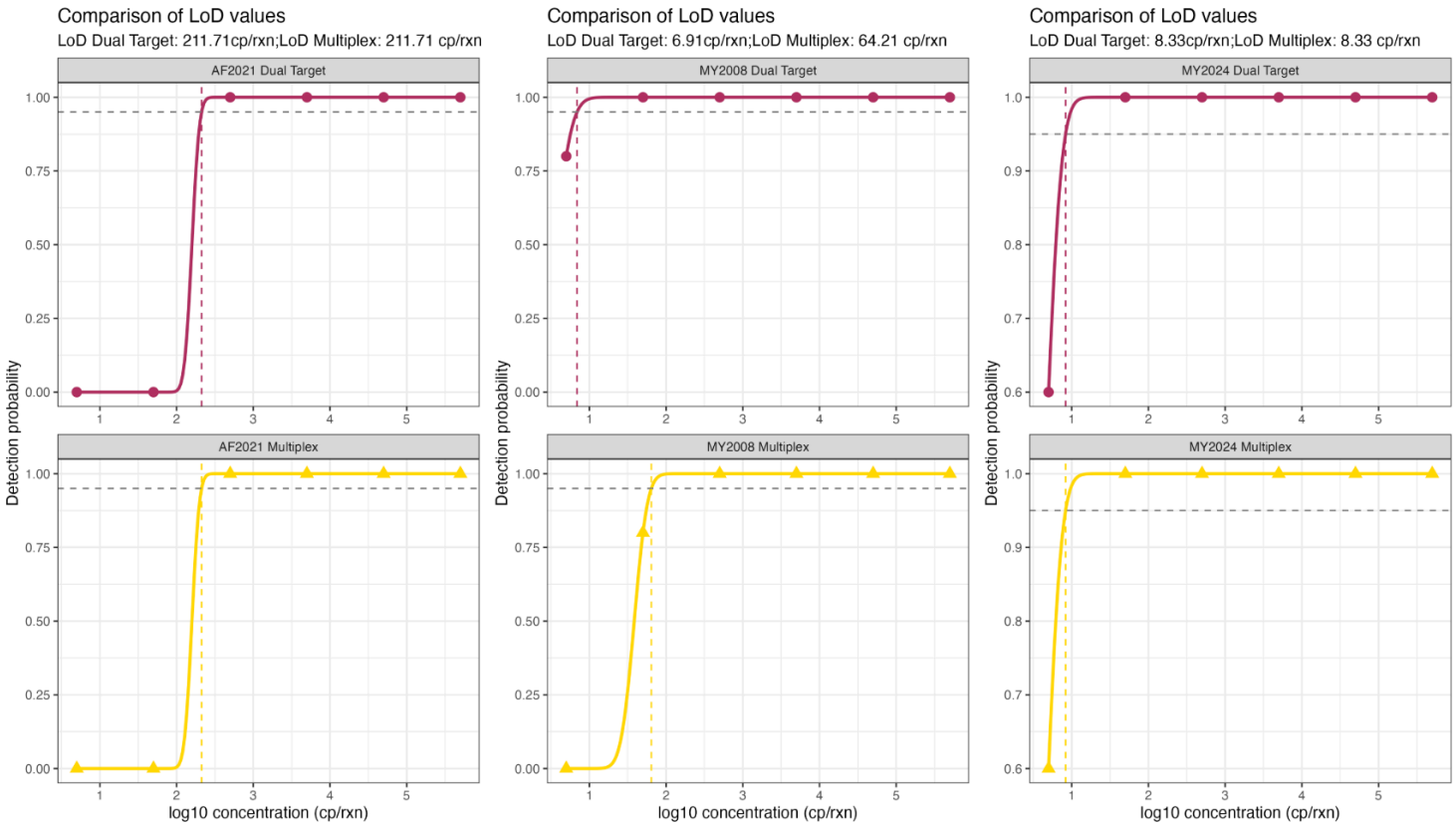


Figure 3

Comparison of the limit of detection (LoD) of the dual-target and multiplex assays for three ivRNA. Detection probability is plotted against viral input (log_{10} copies per reaction) for strains AF2021 (left), MY2008 (middle) and MY2024 (right). The top row shows the dual-target assay (magenta circles) and the bottom row the multiplex assay (yellow triangles). Each point is the proportion of positive replicates at that concentration (n = 5 replicates per dilution, tenfold serial dilutions from 5.10^5 to 5×10^0 cp/rxn. The solid curves are probit regression fits. The grey dashed horizontal line marks 95% detection probability, and the coloured dashed vertical lines mark the LoD, defined as the concentration giving 95% detection. Both assays had the same LoD for AF2021 (211.71 cp/rxn) and MY2024 (8.33 cp/rxn). For MY2008, the dual-target assay was about 9-fold more sensitive than the multiplex assay (6.91 vs. 64.21 cp/rxn).

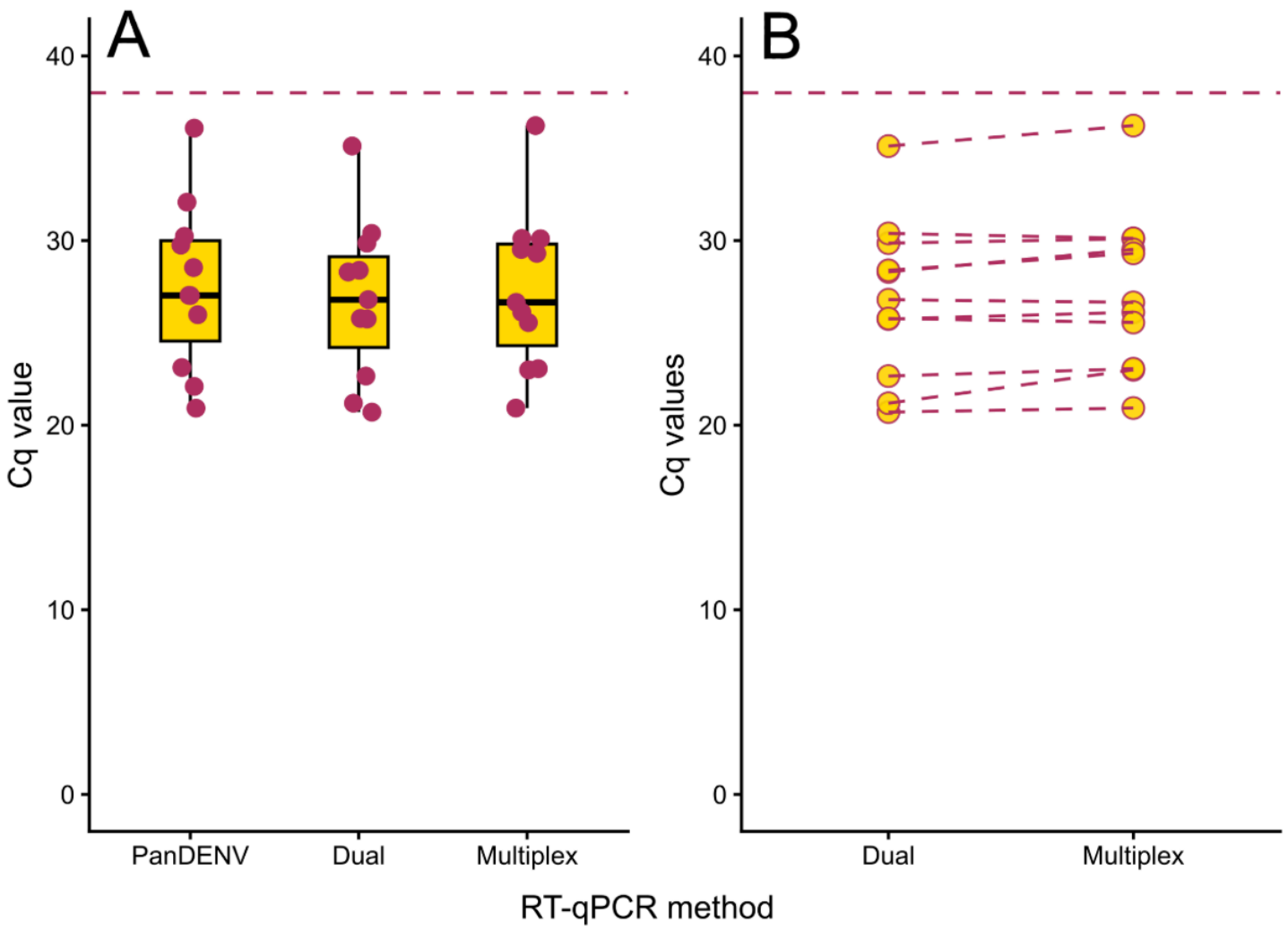


Figure 4

RT-qPCR results of clinical validation samples. A) Distribution of Cq values on clinical validation panel (RNA from DENV-2/GVI infected patient in Kolda in 2021 and spiked serum with DENV-2/GVI viral isolate) with panDENV and Dual vs Multiplex format developed during this study. B) Paired Cq values in dual and multiplex format

Supplementary Files

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- SupplementaryCombinedSylvatic.docx