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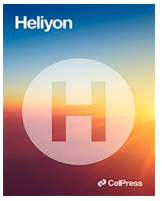

Research article

Broad-spectrum dengue virus detection using the commercial RealStar dengue RT-PCR kit 3.0 (Altona) and an in-house combined real-time RT-PCR assay

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ABSTRACT

In endemic areas, the genetic diversity among co-circulating dengue virus (DENV) strains is considerable and new, highly divergent strains are identified on a regular basis. It is thus critical to ensure that molecular diagnostic tools effectively detect virus genomes even in case of important genetic variation. Here, we tested both the pan-DENV detection capacity and the limit of detection of two real-time RT-PCR assays: (i) the commercial RealStar Altona 3.0 system and (ii) a laboratory developed test (LDT) combining two RT-PCR systems in a single reaction tube (DenAllDUO). We used a panel of DENV strains representative of the genetic diversity within DENV species, combined with three *in vitro* transcribed RNAs as surrogates for unavailable strains corresponding to recently discovered strains with substantial genetic divergence: DENV serotype 1 (DENV-1) Brun2014, DENV-2 QML22 and DENV-4 DKE121. Both systems (i) targeted the genome 3′ untranslated region, (ii) displayed a broad detection spectrum, encompassing most of DENV species diversity, and (iii) detected the three aforementioned divergent strains. DenAllDUO detected all the strains tested, whereas the RealStar system failed to detect strains from DENV-4 genotype III. Altogether, our findings support the value of these two RT-PCR systems as part of the Dengue diagnostic arsenal.

1. Introduction

Dengue virus (DENV) is a single-stranded positive-sense RNA virus belonging to the *Flaviviridae* family, genus *Orthoflavivirus* (formerly *Flavivirus)* [1]. It is the most common arbovirus of human health importance — the WHO estimates that half the population is now exposed to the dengue [2]. Laboratory diagnostic methods for assessing DENV infection rely on the detection of viral RNA, NS1 viral antigen, antibodies, or a combination of these techniques. The molecular detection of the viral genome of the four serotypes of DENV (named DENV-1 to −4) remains pivotal for both diagnostic and epidemiological purposes [3], and can now be achieved using real-time RT-PCR systems and commercial kits. Several studies have evaluated and compared these systems. The literature reports

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several studies evaluating commercial detection tests for DENV. However, these studies do not take into account the evolution of commercial tests some of which, like RealStar Altona, are now available in improved versions. These studies focus mainly on the sensitivity and specificity of tests on clinical samples, and are limited to serotype to characterize strains without genotypic investigations [4–7]. As a result, the "pan-Dengue" detection capacity of the entire DENV genotypic diversity is not assessed. Moreover, highly divergent strains of DENV have recently been reported in the literature. The Brun2014 strain of DENV-1, discovered in 2014 in Brunei, belongs to a sylvatic lineage [8] that has been proposed as a new genotype (VI). In 2015, the highly divergent QML22 strain of DENV-2 identified in Borneo, exhibited a genetic divergence halfway between that observed within the same serotype and that observed between strains of different serotypes [9]. Finally, the DKE121 strain of DENV-4 discovered in 2007 is so divergent that it was proposed as a putative new serotype [10]. Assessing the ability of routine molecular detection assays to identify these highly divergent sequences is crucial to avoid the possible occurrence of false negative results.

Here, we established a panel of DENV strains and *in vitro* transcribed RNAs representative of DENV species genetic diversity at large to test the pan-DENV detection capacity of two real-time RT-PCR systems [11]. The RealStar Dengue RT-PCR Kit 3.0 (Altona) is widely used both in the European university hospitals and in medical biology laboratories (unpublished data from the French arbovirus reference center). The second system is a laboratory developed test (LDT), named “DenAllDUO”, that consists of four primers and two FAM-labeled probes [12,13] targeting the 3’ untranslated region (UTR).

2. Materials and methods

2.1. Panel of 19 DENV strains

We used a panel of 19 DENV strains and 3 *in vitro* transcribed (IVT) RNAs representative of the genetic diversity within DENV species. These strains were obtained from (i) the European Virus Archive, (ii) the French Reference Center for Arboviruses, (iii) the World Reference Center for Emerging Viruses and Arboviruses (Galveston TX, USA). The selection process of the DENV panel has been described previously [14]. Briefly, in order to select representative genotypes, we collected dengue full-length genome sequences from the NCBI database and complemented this database with those, unpublished, of the French National Arbovirus Reference Center (CNR). We performed phylogenetic reconstructions with the maximum likelihood method to assign all available genome sequences to a genotype in a serotype. Within each genotype, we focused on strains that were not subjected to extensive cell passage and were either available as biological isolates in virus collections or as full-length sequences in GenBank. We’ve already used successfully this panel, to challenge antiviral compounds against this diversity [15] (Table 1).

For the 19 strains used for testing, virus stocks had previously been produced in a NSB3 laboratory [14]. Viral RNA was extracted from aliquots of clarified cell culture supernatants using the EZ1 automated system with the Virus 2.0 MiniKit according to the manufacturer’s instruction (both from Qiagen). Nucleic acid extracts were then diluted to reach cycle threshold (CT) values of between 30 and 34 in real-time RT-PCR.

2.2. Generation of in vitro transcribed RNAs (IVT RNAs)

The target regions was included in plasmids synthesized by Genescript (Piscataway NJ, USA). The first plasmid contained the 314

Table 1
List of 19 strains and 3 IVT RNA representing the DENV species genetic diversity.

Serotype	Genotype	Virus strain (GenBank accession no)	IVT RNA encompassing the target region of both assays
DENV-1	**I**	H/IMTSSA/98/606 (AF298808)	
	III	P72-1244 (EF457905)	
	IV	JKT 1186 TVP 949 (EU074031)	
	V	CNR_25329 (MF004384)	
	VI		DENV1-Brun2014 (KR919820)
DENV-2	**American**	1751 TC 544 (EU073981)	
	Asian/American	H/IMTSSA-MART/98–703 (AR208496)	
	Asian I	CNR 25326 (MH888331)	
	Asian II	PG/BID-V2618/2008_PG (FJ906959)	
	Cosmopolitan	CNR_25679 (MF004385)	
	Sylvatic	DKD811 (FJ467493)	
	Unknown		DENV2-QML22 (KX274130)
DENV-3	**I**	CNR 17046 (MF004386)	
	II	CNR15418 (MH888332)	
	III	4025 (MH888333)	
	V	BR/D3LIMHO/2006 (JN697379)	
DENV-4	**I**	G11337 (JF262783)	
	IIa	CNR_16861 (MH888334)	
	IIb	Dakar HD 34460 (MF004387)	
	III	ThD4 0476 97 (AY618988)	
	Sylvatic	P57-514 (JF262779)	
	Unknown		DENV4-DKE121(OK605599)

nucleotides of the 3′UTR region of a DENV-1 (GenBank accession number: MN018297) targeted by the DenAllDUO assay; the second plasmid contained 600 nucleotides of the 3′UTR region of the Brun2014 DENV-1 strain (GenBank accession number: KR919820), the third plasmid contained 600 nucleotides of the 3′UTR region of the QML22 DENV-2 strain (GenBank accession number: KX274130); the fourth plasmid contained 600 nucleotides of the 3′UTR region of the DKE-121 DENV-4 strain (GenBank accession number: OK605599) – plasmid sequences are available in the supplementary data. All the viral sequences contained in these plasmids were flanked at the 5′ end by the T7 promoter sequence. Following amplification, purified PCR products were used to produce *in vitro* transcribed RNAs (IVT RNAs) using the MEGAshortscript™ T7 Transcription Kit (Invitrogen - ThermoFisher Scientific) as previously described [16]. IVT RNAs were serially diluted from 10^8 to 10^2 copies/μL, and dilutions were stored at −80 °C.

2.3. Real-time RT-PCR assays

For the DenAllDUO assay, RT-PCR reactions were performed with the SuperScript® III Platinum One-Step RT-qPCR Kit with ROX (#11732-088, Invitrogen-ThermoFisher Scientific). Primers and probes were pooled together in the same reaction tube. Both probes were labeled with the same dye (FAM). A 25-μL reaction was set up containing 12.5 μL of 2X Reaction Mix, 0.5 μL of Superscript III RT/Platinum Taq Mix and primers and probe (see details in Table 2), at the concentrations specified in Tables 1 and 5 μL of RNA template. Cycling conditions were: 50 °C for 15 min; 95 °C for 2 min; 45 cycles with 15 s at 95 °C and 45 s at 60 °C. The threshold was set automatically by the software.

The RealStar assay was performed using the RealStar dengue PCR kit 3.0 (Altona Diagnostics, Hamburg, Germany). Reaction mixtures were prepared with the recommended volume of 30 μL, with each reaction mixture containing 5 μL of master mix A and 15 μL of master mix B. Ten microliters of RNA template were added to each reaction mixture. RT-PCRs were again performed using the CFX thermal cycler (Biorad) with the following cycling conditions: 55 °C for 20 min (RT step), 95 °C for 2 min, 45 cycles with 15 s at 95 °C, 45 s at 55 °C and 15 s at 72 °C. The threshold was set automatically by the software.

2.4. Analytical sensitivity

The analytical sensitivity was measured using the IVT RNA Duo DENV. Serial dilutions of the quantified IVT RNAs were prepared using the AE buffer (ref 740917.1, Macherey-Nagel™, Hoerdt). Seven concentrations (from 200 to 0.6 copies/μL) were tested, with 8 replicates for each. A cycle threshold (Ct) $\geq$40 was considered negative. The lower limit of detection (LOD) was determined by probit regression analysis, using IBM SPSS Statistics software version 24. The LOD was defined as a concentration of viral copies, achieving a 95 % hit rate (LOD95) according to ISO11843.

2.5. Sequencing of amplicons

Purified amplicons were pooled and then sequenced by next generation sequencing. After Qubit quantification using the Qubit® dsDNA HS Assay Kit and Qubit 2.0 fluorometer (ThermoFisher Scientific), libraries were built adding barcods for sample identification using AB Library Builder System (ThermoFisher Scientific). To pool the barcoded samples equimolarly, a quantification step by real time PCR was carried out using the Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific). Both the emulsion PCR of the pools and loading on 520 chip were achieved using the automated Ion Chef instrument (ThermoFisher). Sequencing was performed using the S5 Ion torrent technology (Thermo Fisher Scientific) following the manufacturer's instructions. Using CLC genomics workbench software v.21.0.5 (Qiagen), reads were trimmed and mapped on a reference and a consensus was called based on this alignment.

3. Results

According to Altona manufacturer's data, the 95 % limit of detection (LOD95) of the RealStar kit is 7.04 copies/μL. We estimated the LOD95 of the DenAllDUO system using a system-specific IVT RNAs derived from a DENV-1 genome (GenBank accession number MN018297) and found a similar value of 11.6 copies/μL (95 % confidence interval: 6.5–56.1 copies/μL).

Regarding the detection spectrum, the DenAllDUO system detected all strains from the panel tested, while the RealStar system

Table 2
In-house DenAllDUO system composition.

Reference	Primer and Probes	5′-3′ sequence	Target	Position[a]	Amplicon size	Concentration nM
Leparc-Goffart et al. [16]	DenALL1-F	AGGACYAGAGGTTAGAGGAGA	3′UTR	10579–10599	106 nt	400
	DenALL1-R	CGYTCTGTGCCTGGAWTGAT		10666–10685		400
	DenALL1-P	FAM-ACAGCATATTGACGCTGGGARAGACC-QSY		10617–10642		200
Huhtamo et al. [17]	DenALL2-F	GGACTAGAGGTTAGAGGAGACCCC	3′UTR	10580–10603	79 nt	900
	DenALL2-R	GAGACAGCAGGATCTCTGGTC		10619–10636		900
	DenALL2-P	FAM-AGCATATTGACGCTGGGA-QSY		10639–10659		250

[a] According to the sequence of Dengue virus 2 (Genbank Accession Number NC_001474.2).

failed to detect ThD4 0476 97 (DENV-4 GIII). Out of the 4 replicates performed for this strain, one was negative and the other three were very weakly positive (mean CT value: 38.7). Sequencing of the amplicons generated with the RealStar system showed that, like to the DenAllDUO system, the commercial system targets the 3′UTR region of DENV genome (Fig. 1).

As presented in Table 3, the ΔCT ([mean CT value with DenAllDUO system] – [mean CT value with RealStar system]) is superior to 0 in 18/22 cases (82 %) and superior to 1 in 12/22 cases (55 %). We used the quantified RNA IVTs mimicking the 3′UTR region of the genome of 3 highly divergent strains of DENV to assess more accurately the LOD95 of the two PCR systems. Results show that the RealStar system has a slightly lower limit of detection than the DenAllDUO system for DENV1-Brun2014 and DENV2-QML22 but not for DENV4-DKE121. Those differences are however not significant, with overlapping 95 % confidence intervals (Table 4 and Supplemental Tables 1 and 2 and supplemental figures 1, 2 and 3).

4. Discussion

Studies that evaluated the version 1.0 of the RealStar Dengue RT-PCR Kit reported a 100 % specificity and a sensitivity between 72 and 89 % based on clinical samples(3,6). We were unable to find any recent data for the version 3.0 used in this study. Sequencing of PCR amplicons indicated that, as with the in-house DenAllDUO system, the primers and probes of this kit target conserved sequence motifs located in the 3′UTR of DENV genome. The results from our study suggest that the RealStar system presents a slightly lower limit of detection than the DenAllDUO system. Comparison of the mean CT values observed for the two systems against our panel of dengue strains suggests a better overall detection for the RealStar system than for the DenAllDUO system. It should be noted that the amount of RNA used in the PCR reaction differs between the two protocols, since twice as much RNA is used in the RealStar assay. However, estimation of LOD95s using three IVTs showed that the difference is not significant due to overlapping confidence intervals.

Both systems showed good pan-DENV detection capacity using viral RNA from a panel of strains representative of DENV diversity and three highly divergent strains of DENV-1, DENV-2 and DENV-4 recently discovered (Fig. 2a). One exception however, was DENV-4 genotype III, which was not detected (or barely detected) by the RealStar system. Although the sequences of the primers and probe(s) for that system are not available, *in silico* analysis did not display significant sequence divergence compared with DENV-4 belonging to other genotypes (Figs. 1 and 2b). This virus was only described in the 2000s in Thailand and there are currently only 5 sequences

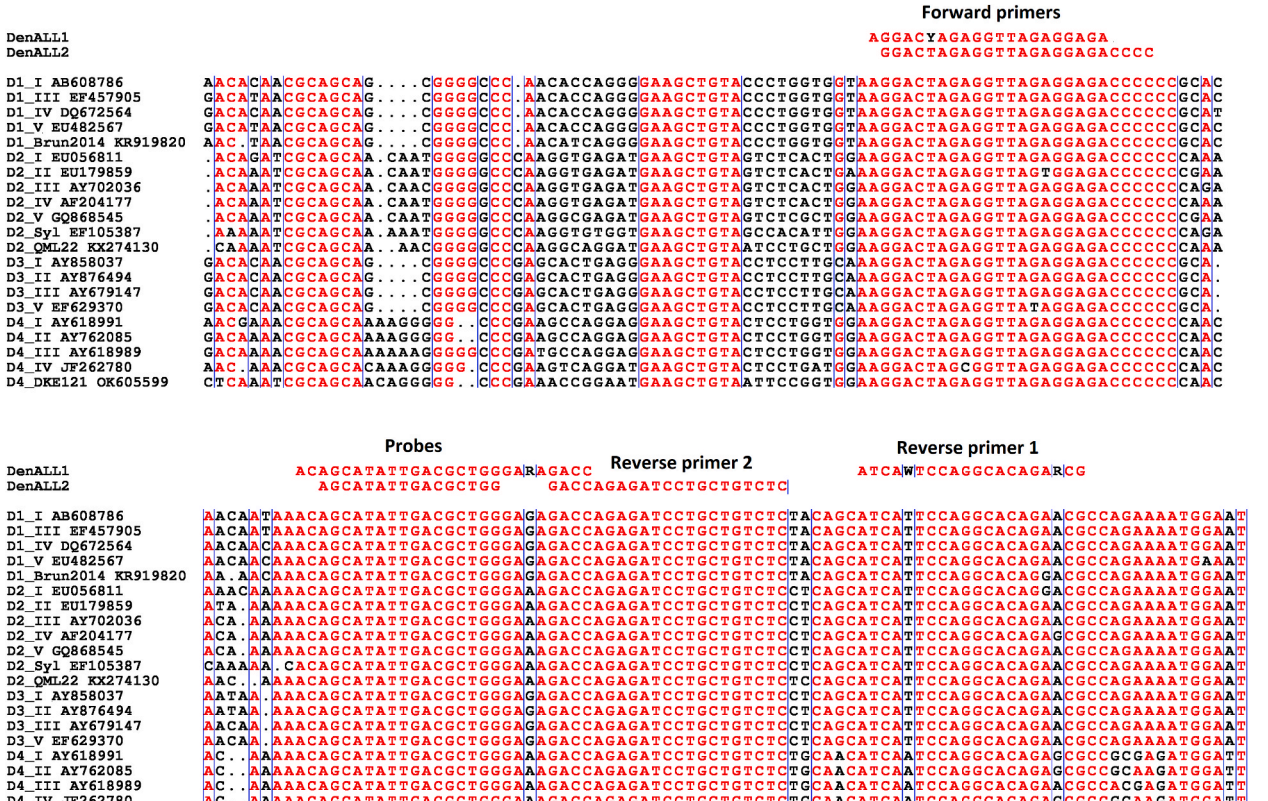

Fig. 1. Alignment of the putative 3′UTR region where the RealStar system is located (estimated by NGS sequencing of the amplicons obtained with the RealStar system for the 4 DENV types). The alignment is reduced to one sequence per genotype (Roman numeral) for each serotype (denoted D1, D2, D3 or D4). Areas in red correspond to nucleotides that are 100 % conserved between the sequences shown. Primers and probes of the two combined RT-PCR systems constituting the DenAllDUO system are represented.

Table 3
List of the 19 strains and 3 IVT RNA used to assess the breadth of the detection spectrum of the two real-time RT-PCR systems. Mean cycle threshold (CT) values were calculated from two independent experiments with two replicates each. *Negative amplification for one replicate (a CT value of 40 was used for mean calculation). The ΔCT corresponds to [mean CT value with DenAllDUO system] – [mean CT value with RealStar system]. SD means standard deviation.

Serotype	Genotype	Name of the viral strain or IVT (GenBank accession nb)	RealStar system	DenAllDUO sytem	ΔCT
			Mean CT values ± SD		
DENV-1	**I**	H/IMTSSA/98/606 (AF298808)	33.1 ± 0.3	33.0 ± 0.5	0.0
	III	P72-1244 (EF457905)	32.6 ± 0.8	32.8 ± 0.7	0.3
	IV	JKT 1186 TVP 949 (EU074031)	33.1 ± 1.0	32.8 ± 0.9	−0.3
	V	CNR_25329 (MF004384)	32.1 ± 1.1	33.2 ± 0.3	1.2
	VI	DENV1-Brun2014 (KR919820)	32.5 ± 0.3	33.3 ± 1.1	0.8
DENV-2	**American**	1751 TC 544 (EU073981)	29.7 ± 0.5	30.5 ± 0.2	0.7
	Asian/American	H/IMTSSA-MART/98–703 (AR208496)	28.9 ± 0.7	31.6 ± 0.3	2.7
	Asian I	CNR 25326 (MH888331)	31.6 ± 0.5	32.7 ± 0.7	1.1
	Asian II	PG/BID-V2618/2008_PG (FJ906959)	31.0 ± 0.9	31.7 ± 0.6	0.6
	Cosmopolitan	CNR_25679 (MF004385)	32.2 ± 0.7	32.2 ± 0.6	0.0
	Sylvatic	DKD811 (FJ467493)	31.5 ± 0.2	31.0 ± 0.4	−0.5
	Unknown	DENV2-QML22 (KX274130)	32.8 ± 0.3	32.4 ± 0.3	−0.36
DENV-3	**I**	CNR 17046 (MF004386)	30.3 ± 0.8	32.2 ± 0.2	1.9
	II	CNR15418 (MH888332)	29.0 ± 1.1	31.0 ± 0.2	2.0
	III	4025 (MH888333)	32.0 ± 0.7	33.3 ± 0.4	1.3
	V	BR/D3LIMHO/2006 (JN697379)	28.4 ± 1.2	31.1 ± 0.2	2.8
DENV-4	**I**	G11337 (JF262783)	30.3 ± 0.1	31.8 ± 0.5	1.5
	IIa	CNR_16861 (MH888334)	28.8 ± 0.5	30.3 ± 0.2	1.5
	IIb	Dakar HD 34460 (MF004387)	29.4 ± 0.4	32.7 ± 0.3	3.3
	III	ThD4 0476 97 (AY618988)	**38.7*** ± 1.3	32.1 ± 0.1	**−6.6**
	Sylvatic	P57-514 (JF262779)	32.1 ± 0.1	31.8 ± 0.5	1.5
	Unknown	DENV4-DKE121(OK605599)	32.4 ± 0.1	33.5 ± 1.4	1.1

Table 4
Results of the different 95 % detection limits (LOD95) estimated for the two real-time RT-PCR systems using three IVT RNAs deriving from the sequence of the 3′UTR of three highly divergent genotype of DENV recently described.

IVT RNAs	LOD95 RealStar® [95 % confidence interval] (copies/μL)	LOD95 DenAllDUO [95 % confidence interval] (copies/μL)
DENV1-Brun2014	5.8 [3.0–85.9]	17.6 [5.7–262.2]
DENV2-QML22	0.7 [0.3–9.9]	2.2 [1.0–14.6]
DENV4-DKE121	68.4 [29.3–497.4]	3.8 [2.5–121.1]

deposited in GenBank including 2 complete genomes in which the 3′UTR region is identical [17]. This discovery shows that for viruses with high genomic diversity, in the event of the emergence of new genotypes, the use of tests with public primer and probe sequences enables rapid *in silico* verification that the system works theoretically. This is not possible with a closed system whose sequences are not known. This also illustrates how the use of combined systems in conserved regions of the genome may be advantageous for overcoming genetic variations.

5. Conclusion

The RealStar Dengue RT-PCR Kit 3.0 and the DenAllDUO system meet the requirements to be part of the dengue diagnostic arsenal. This is the first description for the DenAllDUO which combines two previously evaluated tests and offers the possibility of reliable and inexpensive diagnosis. Both systems present a low limit of detection even for highly divergent genotypes of recent discovery and a broad detection spectrum with the exception of DENV4 genotype III, which could only be amplified by the DenAllDUO system.

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Data availability statement

Data included in article/supp. material/referenced in article.

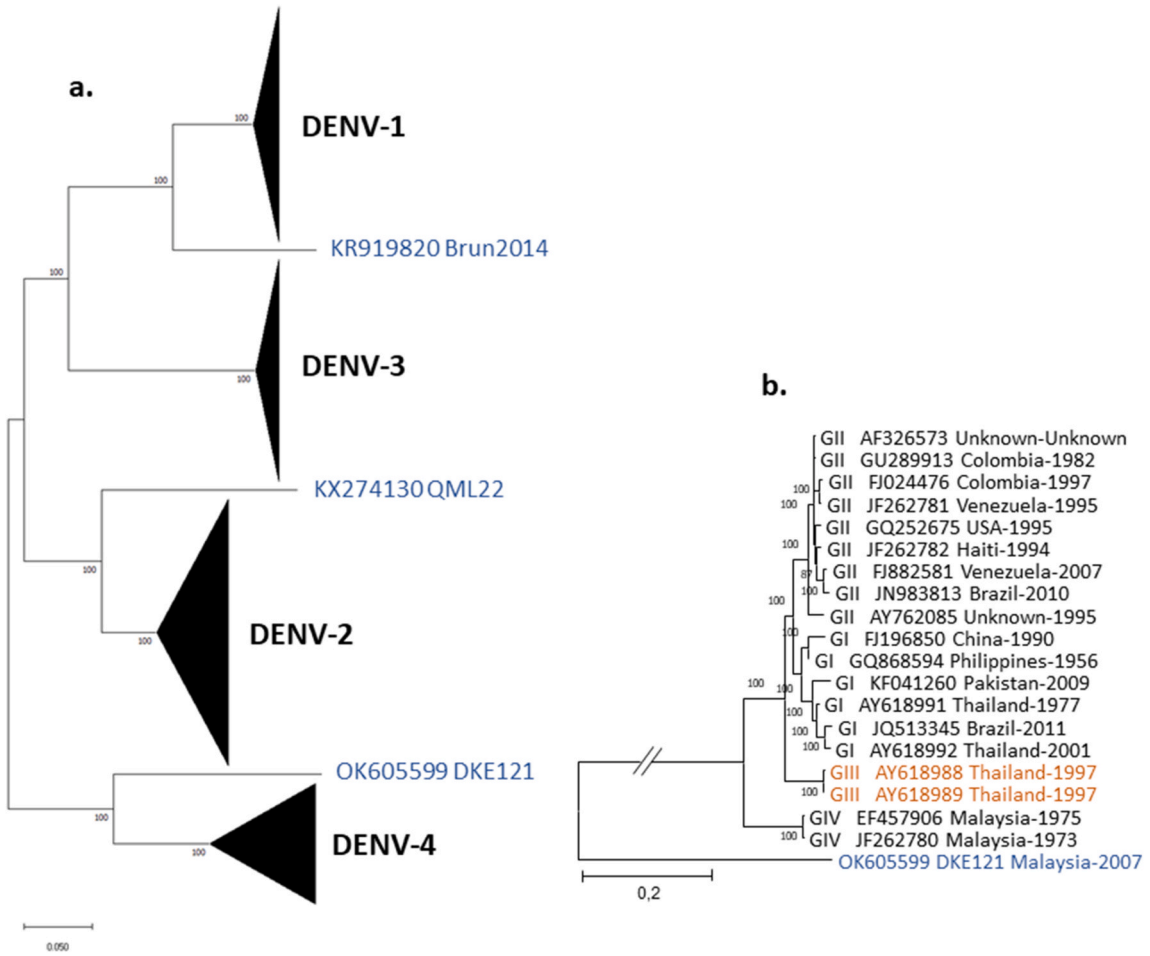

Fig. 2. Phylogenetic tree representing DENV diversity using nucleotide sequences from complete genomes including 136 references [a] and highlighting the divergence of the Brun2014, QML22 and DKE121 strains (in blue with Genbank accession numbers). Tree [b] is a focus on serotype 4 (genotype with Roman numeral, Genbank accession numbers with year and country of isolation) showing genotype III (in orange) not amplified by the RealStar system does not appear to be significantly divergent. Both phylogenetic trees were performed with MEGAX software using Maximum Likelihood method, General Time Reversible model. Numbers represent bootstrap values for each node calculated with 1000 replicates. Bootstraps lower than 80 % are not shown.

CRediT authorship contribution statement

Léa Luciani: Writing – original draft, Methodology, Investigation, Conceptualization. **Pierre Combe:** Writing – original draft, Investigation, Data curation. **Franck Touret:** Writing – review & editing, Supervision, Resources. **Céline Gazin:** Data curation. **Raphaëlle Klitting:** Writing – review & editing, Resources. **Laura Pezzi:** Writing – review & editing. **Laurence Thirion:** Resources, Conceptualization. **Rémi Charrel:** Writing – review & editing, Supervision, Methodology. **Gilda Grard:** Writing – review & editing, Resources. **Xavier de Lamballerie:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Antoine Nougairède:** Writing – review & editing, Supervision, Project administration, Methodology, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31252.

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