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Phylogenetically based establishment of a dengue virus panel, representing all available genotypes, as a tool in dengue drug discovery

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3 Franck Touret^{1*}, Cécile Baronti¹, Olivia Goethals², Marnix Van Loock², Xavier de

4 Lamballerie¹ and Gilles Querat¹

5 ¹: Unité des Virus Émergents (UVE : Aix-Marseille Univ – IRD 190 – Inserm 1207 – IHU
6 Méditerranée Infection), Marseille, France

7 ² : Janssen Infectious Diseases Discovery, Janssen Pharmaceutica NV, Turnhoutseweg 30,
8 2340, Beerse, Belgium.

9 *Corresponding author: franck.touret@hotmail.fr

10

11 **Abstract**

12 Dengue fever is the most widespread of the human arbovirus diseases, with approximately
13 one third of the world's population at risk of infection. Dengue viruses are members of the
14 genus *Flavivirus* (family *Flaviviridae*) and, antigenically, they separate as four closely related
15 serotypes (1-4) that share 60 to 75 % amino acid homology. This genetic diversity
16 complicates the process of antiviral drug discovery. Thus, currently no approved dengue-
17 specific therapeutic treatments are available. With the aim of providing an efficient tool for
18 dengue virus drug discovery, a collection of nineteen dengue viruses, representing the
19 genotypic diversity within the four serotypes, was developed. After phylogenetic analysis of
20 the full-length genomes, we selected relevant strains from the EVAg collection at Aix-
21 Marseille University and completed the virus collection, using a reverse genetic system based
22 on the infectious sub-genomic amplicons technique. Finally, we evaluated this dengue virus
23 collection against three published dengue inhibitory compounds. NITD008, which targets the

24 highly conserved active site of the viral NS5 polymerase enzyme, exhibited similar antiviral
25 potencies against each of the different dengue genotypes in the panel. Compounds targeting
26 less conserved protein subdomains, such as the capsid inhibitor ST-148, or SDM25N, a δ
27 opioid receptor antagonist which indirectly targets NS4B, exhibited larger differences in
28 potency against the various genotypes of dengue viruses. These results illustrate the
29 importance of a phylogenetically based dengue virus reference panel for dengue antiviral
30 research. The collection developed in this study, which includes such representative dengue
31 viruses, has been made available to the scientific community through the European Virus
32 Archive to evaluate novel DENV antiviral candidates.

33 **Keywords**

34 Dengue virus; phylogenetic analysis; reverse genetic; drug discovery; virus panel; dengue inhibitors

35 **Short communication**

36 Dengue virus (DENV) is a major threat to human health, with approximately one third of the
37 world's population at risk of being infected. DENV is the causative agent of dengue fever, as
38 well as the more severe dengue haemorrhagic fever (DHF)(Messina et al., 2014) and dengue
39 shock syndrome (DSS). It belongs to the genus *Flavivirus* (*Flaviviridae* family), which
40 comprises other clinically important human pathogens, such as yellow fever virus, West Nile
41 virus and the recently emerging Zika virus(Vasilakis and Weaver, 2017). DENV is an
42 arthropod borne virus transmitted through the bite of infected mosquitoes from the genus
43 *Aedes* (*Stegomyia*). Epidemiological transmission of DENV is confined to urban and peri-
44 urban cycles for which *Aedes aegypti* and *Ae albopictus* mosquitoes, respectively, are the
45 primary transmission vectors(Chen and Vasilakis, 2011). Dengue is a positive-sense single
46 stranded RNA virus with a 10.7 kb genome encoding a single polyprotein which is post-
47 translationally processed into three structural proteins, viz., capsid (C), pre-membrane (prM),

48 envelope (E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and
49 NS5)(Gebhard et al., 2011). Four antigenically closely related serotypes of DENV (1-4)
50 which share 60 to 75 % amino acid homology, have been identified(Guzman and Harris,
51 2015). Within this serotype demarcation, the DENV are also grouped into genotypes, with
52 varying terminology between authors(Carrillo-Valenzo et al., 2010; Weaver and Vasilakis,
53 2009) (hereunder we refer to the grouping proposed by Weaver and Vasilakis(Weaver and
54 Vasilakis, 2009)).

55 Hence, many of the DENV diagnostic tools do not readily distinguish between DENV
56 serotypes. Moreover, co-circulation of different serotypes during DENV epidemics(Vilela et
57 al., 2016) increases the complexity of virus identification. Added to these factors, antibody
58 dependent enhancement of the disease i.e., when patients contract a heterotypic secondary
59 DENV infection(Katzelnick et al., 2017) is a potential additional complication for effective
60 treatment of patients. Consequently, scientists are faced with the challenge of developing
61 Directly Active Antivirals (DAA) that can inhibit the entire spectrum of genetically diverse
62 serotypes and/or genotypes of DENV. However, despite the tireless efforts to provide an
63 antiviral therapy(Canard, 2012; Coutard et al., 2008; van Cleef et al., 2013; Yin et al., 2009),
64 there are still no approved drugs on the market to treat dengue infections. At present, the
65 treatments available are merely supportive(Kaptein and Neyts, 2016).

66 A major barrier to evaluating the activity spectrum of potential DENV-inhibitory molecules
67 arises from the non-availability of a well-defined panel of viruses that specifically represents
68 the genetic variability of all characterised DENV isolates. With the aim of providing a tool for
69 DENV research, with which to assess the antiviral activity of potential inhibitory molecules,
70 we have developed a collection of DENV with sequences that include representative
71 genotypes from within the four DENV serotypes (figure 1). Wherever possible, we selected
72 clinical strains with a limited number of passages in cell culture. Strains were selected from

73 either the European Virus Archive (EVA) collection(Romette et al., 2018), the French
74 National Reference Centre for arboviruses (CNR), or the World Reference Center for
75 Emerging Viruses and Arboviruses (WRCEVA). Viruses that could not be obtained but for
76 which full length genome sequences were available, were re-created using the versatile
77 infectious sub-genomic amplicons (ISA) reverse genetics technology(Aubry et al., 2015,
78 2014).

79 In order to select representative genotypes, we collected dengue full-length genome sequences
80 from the NCBI database and complemented this database with those of our, still unpublished,
81 “in house” and CNR strains. We performed phylogenetic reconstructions with the maximum
82 likelihood method to assign all available genome sequences to a genotype in a serotype
83 (supplementary material Fig 1, 2, 3 and 4). Within each genotype, we focused on strains that
84 were not subjected to extensive cell passage and were either available as biological isolates in
85 virus collections or as full-length sequences in GenBank. Six dengue genotypes were
86 available only as complete genome sequences in the NCBI database without any biological
87 strain counterparts in referenced collections (DENV-1 genotype III, DENV-2 genotype
88 sylvatic and Asian II, DENV-3 genotype V and DENV-4 genotype III and sylvatic). Two
89 genotypes were not available at all because of incomplete genome sequence (DENV-1
90 genotype II and DENV-3 genotype IV). To obtain the biological viruses from the completely
91 sequenced strains, we designed reverse genetics systems based on the ISA technique(Atieh et
92 al., 2016; Aubry et al., 2015, 2014) and generated synthetic overlapping DNA fragments that
93 covered each of the entire genome, bordered by a CMV promoter on the 5’ end and a
94 Ribozyme and poly-adenylation signal on the 3’ end. The overlapping fragments were co-
95 transfected into a mix of human and hamster embryonic kidney cell lines (HEK 293 and
96 BHK-21 purchased from the American Cell Culture Collection). This enabled us to recover
97 the missing biological strains to complete the collection. The initial viral stocks were

98 amplified in Vero E6 cells and fully sequenced. All the DENV strains used, have been made
99 available through the EVAg collection (<https://www.european-virus-archive.com/>).

100 Various specific dengue inhibitors that target several viral proteins involved in different
101 replication steps, have been discovered. ST-148, an inhibitor targeting the capsid structural
102 protein, has been reported to inhibit all DENV serotypes in cell culture, although with varying
103 efficiency. This inhibitor also appears promising in the AG-129 mouse model when infected
104 with a strain of DENV-2(Byrd et al., 2013). NITD008, an adenosine analogue inhibitor that
105 targets the RNA-dependent RNA polymerase activity, was shown to be inhibitory against all
106 dengue serotypes as well as other flaviviruses, including West Nile virus, yellow fever virus
107 and tick-borne Powassan virus(Yin et al., 2009). SDM25N, a δ opioid receptor antagonist, has
108 been reported to target the NS4B protein, probably indirectly through a cellular factor. Thus
109 far, it has only been shown to be active against a DENV-2 strain(van Cleef et al., 2013).

110 Based on the different mechanisms of action of the 3 compounds, their respective target and
111 its associated sequence variability across the different genotypes, we hypothesize that the
112 antiviral activity of the compounds might differ between all of the genotypes of DENV.
113 Therefore, the antiviral activity of these three compounds was assessed using a single
114 common protocol based on a viral RNA yield reduction assay(Delang et al., 2016). The assay
115 did not depend on the cytopathogenic potential of the strain, thus allowing for the inclusion of
116 any dengue strain in the panel tested. Because all these strains differed in their replication
117 kinetics, prior to the assay, all DENV MOI and times of readout of the assay were calibrated
118 **so that** the replication growth were still in the log growth curve at time of the collection of the
119 supernatant. Although the maximum reduction of virus yield may depend of the specific strain
120 and assay conditions, the half inhibitory doses (IC50s) are not expected to be affected in these
121 settings and will depend only on the inhibitor efficiency. The compounds were assayed from
122 10 to 0.004 μ M, with 3-fold step-dilution in triplicate. The amount of viral RNA in the

123 supernatant medium, sampled at pre-determined time in the growth cycle, was quantified by
124 qRT-PCR to determine the 50% maximal effective concentration (EC₅₀) (Table 1).

125 The DENV strains of the collection showed similar sensitivity towards the nucleoside
126 analogue inhibitor NITD008 with EC₅₀'s ranging from 0.2μM to 2.8μM, which is in
127 accordance with previously published results (Xie et al., 2015).

128 The capsid inhibitor ST-148 inhibited all DENV-2 genotypes with EC₅₀'s ranging from 0.25
129 to 1.1μM. However, only one genotype of DENV-1 (DENV-1 GIII at 0.5μM), and one of
130 DENV-4 (DENV-4 GIII at 0.3μM), were inhibited by this compound. Finally, no activity was
131 observed against our DENV-3 genotypes, with all EC₅₀'s > 10μM. Although Byrd and co-
132 workers(Byrd et al., 2013) found that the DENV-2 serotype was the most sensitive serotype to
133 this capsid inhibitor and showed up to two log of variability in the inhibition against other
134 serotypes, they did not fully evaluate the variation in susceptibility to other serotypes
135 sufficiently comprehensively to draw conclusions. In their study, they associated ST-148
136 resistance to a Leucine at position 34 instead of a Serine in DENV-2. However, looking at
137 capsid amino-acid alignment of all our DENV panel and their study's strains, and regardless
138 of their sensibility to ST-148, all strains exhibited a Serine at position 34 except DENV-2
139 from Trinidad (1751 TC 544), which presented a Proline at this position, as the Modoc virus
140 that they reported to be sensitive to ST-148 (Byrd et al., 2013). Thus, if resistance for ST-148
141 can arise from S34L mutations in some DENV-2 strains it is clear that it cannot be
142 unequivocally associated to a Leucine in position 34 in other serotypes and genotypes. This
143 suggests that other residues or domains in the capsid protein may be involved in the
144 interaction.

145 SDM25N showed moderate efficacy, with EC₅₀'s ranging from 1.7 – 7.7 μM against a large
146 proportion of the DENV-2 genotype strains, and half of the DENV-1 genotypes. However, no

147 activity was observed against any of the DENV-3 and 4 genotypes, as EC₅₀ were all above 10
148 µM. This result suggests that the binding affinity of NS4B to the hypothetical cellular factor
149 targeted by SDM25N varies greatly among various DENV genotypes and/or that this cellular
150 factor might be dispensable for efficient replication of some DENV genotypes.

151 Overall, the results demonstrate that compounds targeting highly conserved sites, exemplified
152 by nucleoside analogue inhibitor NITD008 (targeting the active site of the polymerase), had a
153 broader pan-serotypic activity, with similar EC₅₀'s regardless of the DENV genotype. In
154 contrast, compounds targeting less conserved proteins or protein subdomains, either directly
155 (*e.g.* the capsid) or indirectly through an interaction with a host factor of the cell (*e.g.*
156 SDM25N), exhibited larger differences in activity towards the various genotypes of DENV.

157 Importantly, these data illustrate the fact that a sound *in cellulo* evaluation of anti-dengue
158 candidate molecules requires the use of a complete reference virus panel that enables
159 estimates of the antiviral activity against each of the identified DENV genotypes to be
160 obtained. Modern reverse genetics techniques have enabled us to develop such a
161 representative collection, and it has been made available to the scientific community through
162 the European Virus Archive collection (EVA). We believe that the availability of this new
163 tool will enable the independent assessment of pan-serotypic activity of anti-dengue
164 candidates in the future, fulfilling a critical requirement for a successful dengue antiviral
165 small molecule.

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170 [virusarchive.com](http://www.european-virusarchive.com)).

171 **Contributions**

172 OG, MVL, GQ and XDL generated the idea of the panel. FT, XDL and GQ conceived the
173 experiments. XDL proposed the study design. FT, CB, and GQ performed the experiments.
174 FT and GQ analysed the results. FT and GQ wrote the paper. FT, CB, GQ, OG, MVL and
175 XDL reviewed and edited the paper.

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271

272 **Table 1 Dengue virus collection-susceptibility to three antiviral compounds assessed by yield**
 273 **reduction assay.** Anti-capsid ST-148, nucleoside analogue NITD008 and δ opioid receptor antagonist
 274 SDM25N were independently tested twice, with 3 replicates per experiment, against the dengue
 275 collection from 10 μ M to 0.005 μ M. AA: Asian American, A: American, C: cosmopolitan.

	Virus	Genotype	ST-148	NITD008	SDM25N
			EC50 (μ M)	EC50 (μ M)	EC50 (μ M)
Dengue 1	D1/H/IMTSSA/98/606 Djibouti	I	>10	0,9 \pm 0,1	>10
	JKT 1186 TYP 949 Indonesia	IV	>10	0,3 \pm 0,03	5,5 \pm 3,67
	CNR_25329 France	V	>10	2,7 \pm 4	7,4 \pm 0,04
	P72-1244 Malaysia	III	3 \pm 0,5	0,9 \pm 0,2	>10
Dengue 2	H/IMTSSA-MART/98-703 France	AA	0,8 \pm 0,5	0,9 \pm 0,3	2,9 \pm 0,95
	_1751 TC 544 Trinidad	A	1 \pm 0,7	0,3 \pm 0,06	2,9 \pm 0,01
	CNR_25679 France	C	1,1 \pm 0,3	0,2 \pm 0,07	1,9 \pm 0,03
	CNR 25326 Thailand	Asian I	0,1 \pm 0,03	0,9 \pm 0,2	7,7 \pm 0,04
	DENV-2/PG/BID-V2618/2008 Papua New Guinea	Asian II	0,2 \pm 0,16	0,3 \pm 0,5	4,1 \pm 0,02
DKD811 Malaysia	Sylvatic	0,4 \pm 0,18	0,4 \pm 0,1	>10	
Dengue 3	DENV3/BR/D3LIMHO/2006 Brazil	V	>10	1 \pm 0,09	>10
	4025 Bolivia	III	>10	1 \pm 0,05	>10
	CNR 17046 Malaysia	I	>10	2,8 \pm 0,3	>10
	CNR 15418 Thailand	II	>10	1,2 \pm 0,3	>10
Dengue 4	G11337 India	I	>10	1,2 \pm 0,03	>10
	Dakar HD 34460 Senegal	IIb	>10	0,9 \pm 0,3	>10
	CNR_16861 Malaysia	IIa	>10	0,4 \pm 0,01	>10
	ThD4_0476_97 Thailand	III	0,3 \pm 0,08	0,2 \pm 0,08	>10
	P75-514 Malaysia	Sylvatic	>10	1 \pm 0,05	>10

276

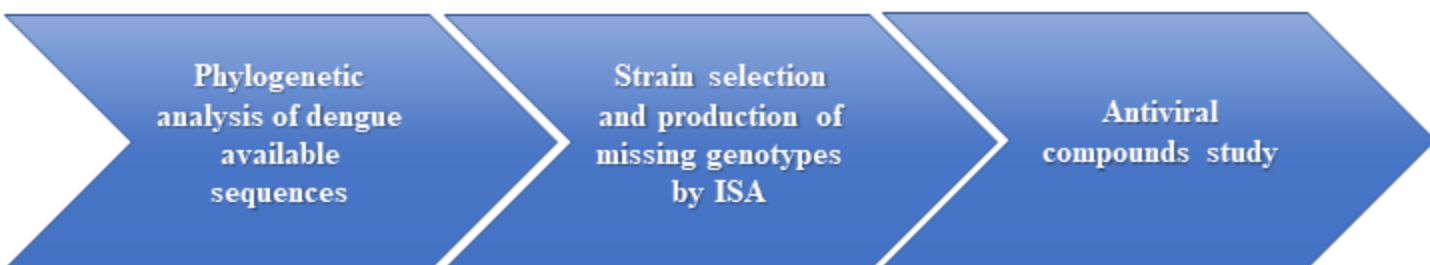
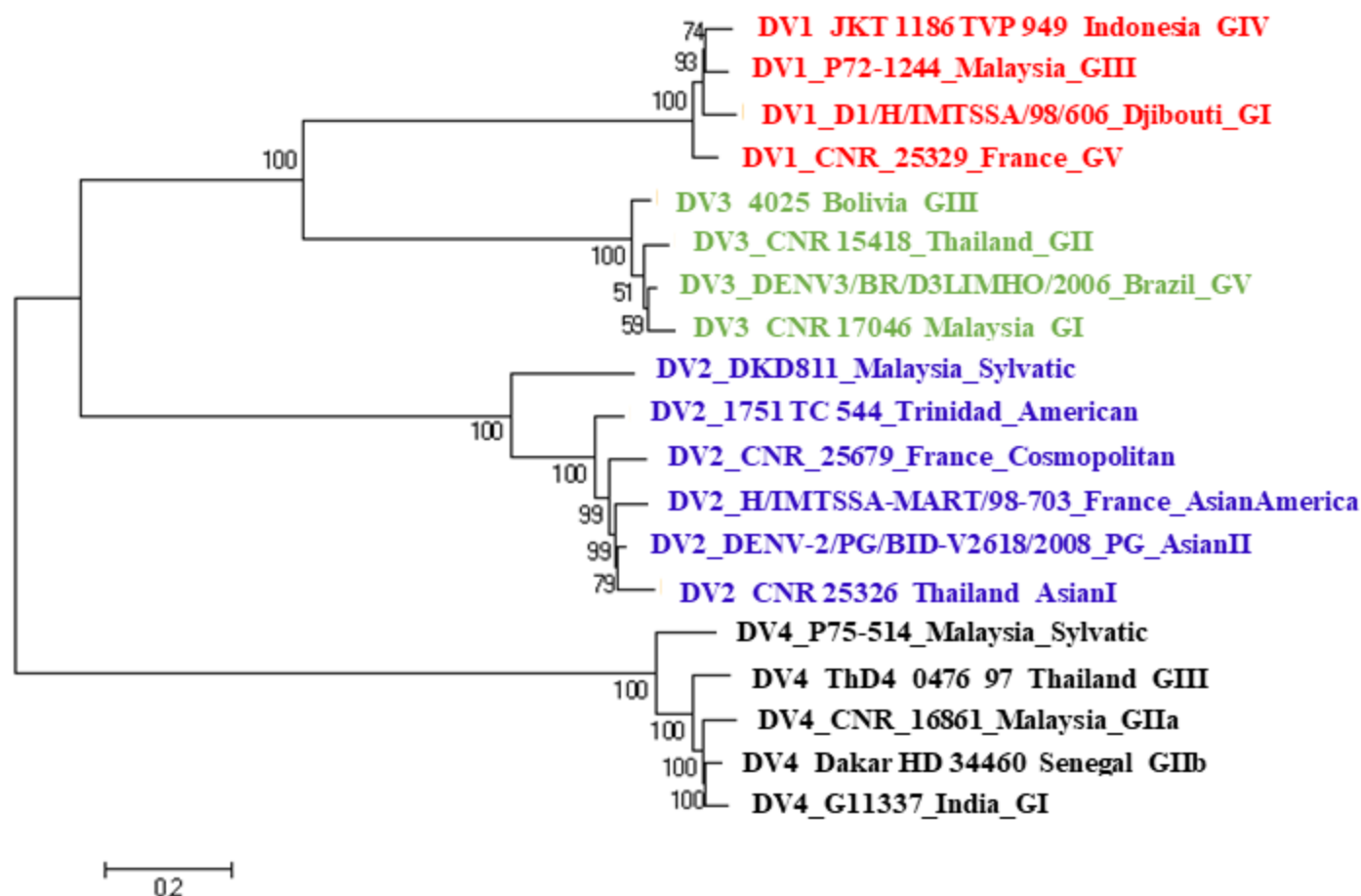
A**B**

Figure 1 : Global serotype-representative DENV collection A: Pipeline of the workflow employed for the virus collection. B: Maximum likelihood phylogenetic tree (GTR+G+I model with 500 bootstraps), based on the complete nucleic acid sequences of the virus collection. Strain information: DENV-1: Genotype I: (Djibouti, 1998, AF298808); Genotype III (Malaysia, 1972, EF457905.1); Genotype IV (Indonesia, 1977, EUO74031); Genotype V (France, 2014, MF004384); DENV-2: Genotype Asian-America (France Martinique, 1998, AF208496); Genotype American (Trinidad, 1953, EU073981.1); Genotype Cosmopolitan (France, 2014, MF004385); Genotype Asian 1 (Thailand, 2014, MH888331); Genotype Asian 2 (Papua New Guinea, 2008, FJ906959.1); Genotype Sylvatic (Malaysia, 2008, FJ467493.1); DENV-3: Genotype I (Malaysia, 2012, MF004386); Genotype II (Thailand, 2012, MH888332); Genotype III (Bolivia, 2011, MH888333); Genotype V (Brazil, 2006, JN697379.1); DENV-4: Genotype IIb (Senegal, 1981, MF004387); Genotype IIa (Malaysia, 2013, MH888334); Genotype III (Thailand, 1997, AY618988.1); Genotype Sylvatic (Malaysia, 1975, JF262779.1); Genotype I (INDIA, 1961, JF262783.1). Complete information relevant to the strains of the collection are more fully detailed in the supplemental material.