Antiviral Research 168 (2019) 109–113

Contents lists available at ScienceDirect

Antiviral Research

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

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

Franck Touret[a,*], Cécile Baronti[a], Olivia Goethals[b], Marnix Van Loock[b], Xavier de Lamballerie[a], Gilles Querat[a]

[a] Unité des Virus Émergents (UVE: Aix-Marseille Univ – IRD 190 – Inserm 1207 – IHU Méditerranée Infection), Marseille, France
[b] Janssen Infectious Diseases Discovery, Janssen Pharmaceutica NV, Turnhoutseweg 30, 2340, Beerse, Belgium

ARTICLE INFO

Keywords:
Dengue virus
Phylogenetic analysis
Reverse genetic
Drug discovery
Virus panel
Dengue inhibitors

ABSTRACT

Dengue fever is the most widespread of the human arbovirus diseases, with approximately one third of the world's population at risk of infection. Dengue viruses are members of the genus *Flavivirus* (family *Flaviviridae*) and, antigenically, they separate as four closely related serotypes (1–4) that share 60–75% amino acid homology. This genetic diversity complicates the process of antiviral drug discovery. Thus, currently no approved dengue-specific therapeutic treatments are available. With the aim of providing an efficient tool for dengue virus drug discovery, a collection of nineteen dengue viruses, representing the genotypic diversity within the four serotypes, was developed. After phylogenetic analysis of the full-length genomes, we selected relevant strains from the EVAg collection at Aix-Marseille University and completed the virus collection, using a reverse genetic system based on the infectious sub-genomic amplicons technique. Finally, we evaluated this dengue virus collection against three published dengue inhibitory compounds. NITD008, which targets the highly conserved active site of the viral NS5 polymerase enzyme, exhibited similar antiviral potencies against each of the different dengue genotypes in the panel. Compounds targeting less conserved protein subdomains, such as the capsid inhibitor ST-148, or SDM25N, a ∂ opioid receptor antagonist which indirectly targets NS4B, exhibited larger differences in potency against the various genotypes of dengue viruses. These results illustrate the importance of a phylogenetically based dengue virus reference panel for dengue antiviral research. The collection developed in this study, which includes such representative dengue viruses, has been made available to the scientific community through the European Virus Archive to evaluate novel DENV antiviral candidates.

1. Short communication

Dengue virus (DENV) is a major threat to human health, with approximately one third of the world's population at risk of being infected. DENV is the causative agent of dengue fever, as well as the more severe dengue haemorrhagic fever (DHF)(Messina et al., 2014) and dengue shock syndrome (DSS). It belongs to the genus *Flavivirus* (*Flaviviridae* family), which comprises other clinically important human pathogens, such as yellow fever virus, West Nile virus and the recently emerging Zika virus(Vasilakis and Weaver, 2017). DENV is an arthropod borne virus transmitted through the bite of infected mosquitoes from the genus *Aedes* (Stegomyia). Epidemiological transmission of DENV is confined to urban and peri-urban cycles for which *Aedes aegypti* and *Ae albopictus* mosquitoes, respectively, are the primary transmission vectors(Chen and Vasilakis, 2011). Dengue is a positive-sense single stranded RNA virus with a 10.7 kb genome encoding a single polyprotein which is post-translationally processed into three structural proteins, viz., capsid (C), pre-membrane (prM), envelope (E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Gebhard et al., 2011). Four antigenically closely related serotypes of DENV (1–4) which share 60–75% amino acid homology, have been identified(Guzman and Harris, 2015). Within this serotype demarcation, the DENV are also grouped into genotypes, with varying terminology between authors(Carrillo-Valenzo et al., 2010; Weaver and Vasilakis, 2009) (hereunder we refer to the grouping proposed by Weaver and Vasilakis(Weaver and Vasilakis, 2009)).

Hence, many of the DENV diagnostic tools do not readily distinguish between DENV serotypes. Moreover, co-circulation of different serotypes during DENV epidemics(Vilela et al., 2016) increases the complexity of virus identification. Added to these factors, antibody dependent enhancement of the disease i.e., when patients contract a heterotypic secondary DENV infection(Katzelnick et al., 2017) is a

* Corresponding author.
E-mail address: franck.touret@hotmail.fr (F. Touret).

https://doi.org/10.1016/j.antiviral.2019.05.005
Received 13 March 2019; Received in revised form 9 May 2019; Accepted 10 May 2019
Available online 11 May 2019

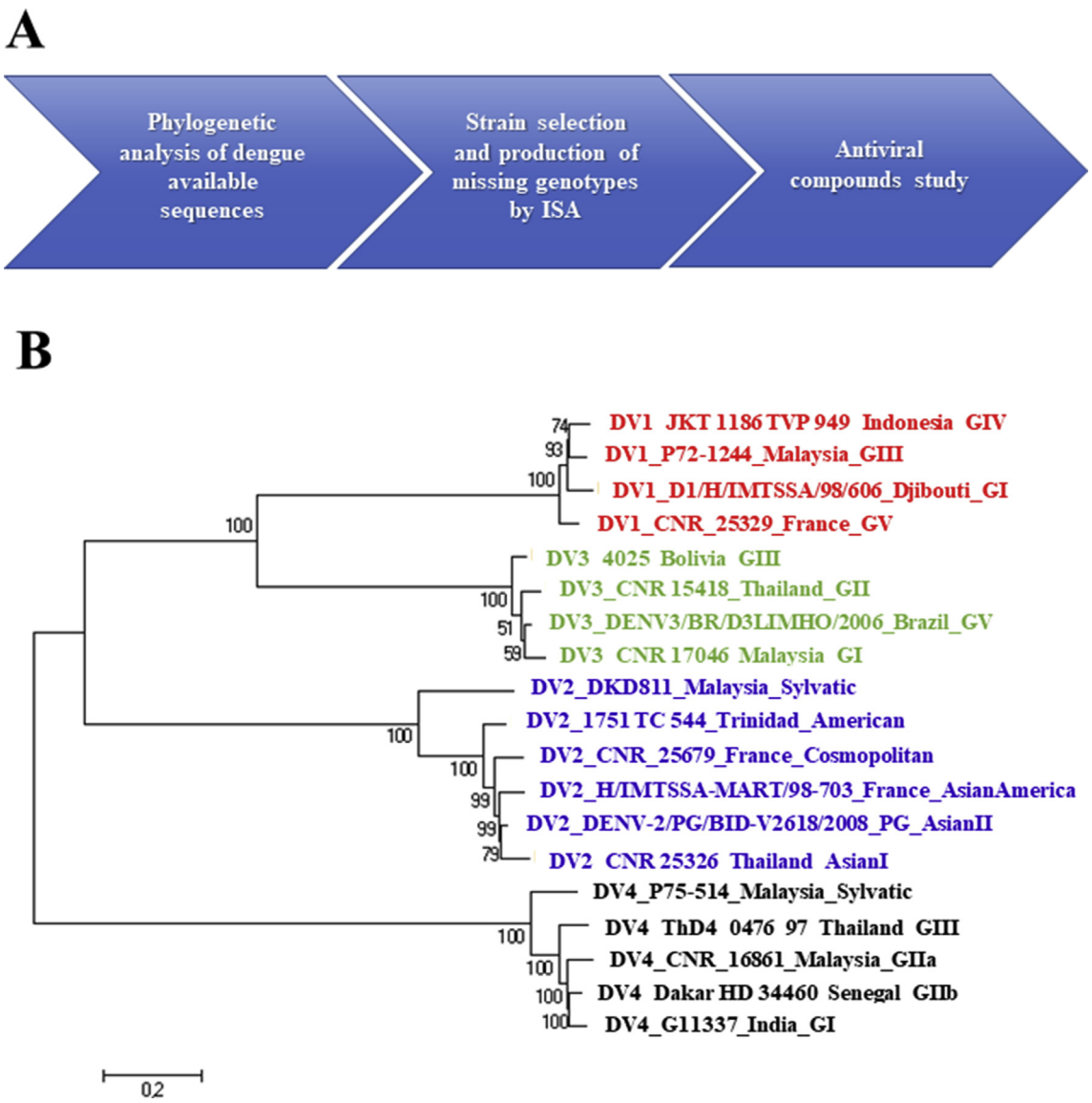

Fig. 1. Global serotype-representative DENV collection A: Pipeline of the workflow employed for the virus collection. **B:** Maximum likelihood phylogenetic tree (GTR + G + I model with 500 bootstraps), based on the complete nucleic acid sequences of the virus collection. Strain information: DENV-1: 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..

potential additional complication for effective treatment of patients. Consequently, scientists are faced with the challenge of developing Directly Active Antivirals (DAA) that can inhibit the entire spectrum of genetically diverse serotypes and/or genotypes of DENV. However, despite the tireless efforts to provide an antiviral therapy(Canard, 2012; Coutard et al., 2008; van Cleef et al., 2013; Yin et al., 2009), there are still no approved drugs on the market to treat dengue infections. At present, the treatments available are merely supportive(Kaptein and Neyts, 2016).

A major barrier to evaluating the activity spectrum of potential DENV-inhibitory molecules arises from the non-availability of a well-defined panel of viruses that specifically represents the genetic variability of all characterised DENV isolates. With the aim of providing a tool for DENV research, with which to assess the antiviral activity of potential inhibitory molecules, we have developed a collection of DENV with sequences that include representative genotypes from within the four DENV serotypes (Fig. 1). Wherever possible, we selected clinical strains with a limited number of passages in cell culture. Strains were selected from either the European Virus Archive (EVA) collection (Romette et al., 2018), the French National Reference Centre for arboviruses (CNR), or the World Reference Centre for Emerging Viruses and Arboviruses (WRCEVA). Viruses that could not be obtained but for which full length genome sequences were available, were re-created using the versatile infectious sub-genomic amplicons (ISA) reverse genetics technology(Aubry et al., 2015, 2014).

In order to select representative genotypes, we collected dengue full-length genome sequences from the NCBI database and complemented this database with those of our, still unpublished, “in house” and CNR strains. We performed phylogenetic reconstructions with the maximum likelihood method to assign all available genome sequences

to a genotype in a serotype (Supplementary Material Figs. 1, 2, 3 and 4). 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. Six dengue genotypes were available only as complete genome sequences in the NCBI database without any biological strain counterparts in referenced collections (DENV-1 genotype III, DENV-2 genotype sylvatic and Asian II, DENV-3 genotype V and DENV-4 genotype III and sylvatic). Two genotypes were not available at all because of incomplete genome sequence (DENV-1 genotype II and DENV-3 genotype IV). To obtain the biological viruses from the completely sequenced strains, we designed reverse genetics systems based on the ISA technique(Atieh et al., 2016; Aubry et al., 2015, 2014) and generated synthetic overlapping DNA fragments that covered each of the entire genome, bordered by a CMV promoter on the 5′ end and a Ribozyme and poly-adenylation signal on the 3' end. The overlapping fragments were co-transfected into a mix of human and hamster embryonic kidney cell lines (HEK 293 and BHK-21 purchased from the American Cell Culture Collection). This enabled us to recover the missing biological strains to complete the collection. The initial viral stocks were amplified in Vero E6 cells and fully sequenced. All the DENV strains used, have been made available through the EVAg collection (https://www.european-virus-archive.com/).

Various specific dengue inhibitors that target several viral proteins involved in different replication steps, have been discovered. ST-148, an inhibitor targeting the capsid structural protein, has been reported to inhibit all DENV serotypes in cell culture, although with varying efficiency. This inhibitor also appears promising in the AG-129 mouse model when infected with a strain of DENV-2(Byrd et al., 2013). NITD008, an adenosine analogue inhibitor that targets the RNA-dependent RNA polymerase activity, was shown to be inhibitory against all dengue serotypes as well as other flaviviruses, including West Nile virus, yellow fever virus and tick-borne Powassan virus(Yin et al., 2009). SDM25N, a ∂ opioid receptor antagonist, has been reported to target the NS4B protein, probably indirectly through a cellular factor. Thus far, it has only been shown to be active against a DENV-2 strain (van Cleef et al., 2013). Based on the different mechanisms of action of the 3 compounds, their respective target and its associated sequence variability across the different genotypes, we hypothesize that the antiviral activity of the compounds might differ between all of the genotypes of DENV. Therefore, the antiviral activity of these three compounds was assessed using a single common protocol based on a viral RNA yield reduction assay(Delang et al., 2016). The assay did not depend on the cytopathogenic potential of the strain, thus allowing for the inclusion of any dengue strain in the panel tested. Because all these strains differed in their replication kinetics, prior to the assay, all DENV MOI and times of readout of the assay were calibrated so that the replication growth were still in the log growth curve at time of the collection of the supernatant. Although the maximum reduction of virus yield may depend of the specific strain and assay conditions, the half inhibitory doses (IC50s) are not expected to be affected in these settings and will depend only on the inhibitor efficiency. The compounds were assayed from 10 to 0.004 μM, with 3-fold step-dilution in triplicate. The amount of viral RNA in the supernatant medium, sampled at pre-determined time in the growth cycle, was quantified by qRT-PCR to determine the 50% maximal effective concentration (EC_{50}) (Table 1).

The DENV strains of the collection showed similar sensitivity towards the nucleoside analogue inhibitor NITD008 with EC_{50}'s ranging from 0.2 μM to 2.8 μM, which is in accordance with previously published results (Xie et al., 2015).

The capsid inhibitor ST-148 inhibited all DENV-2 genotypes with EC_{50}'s ranging from 0.25 to 1.1 μM. However, only one genotype of DENV-1 (DENV-1 GIII at 0.5 μM), and one of DENV-4 (DENV-4 GIII at 0.3 μM), were inhibited by this compound. Finally, no activity was observed against our DENV-3 genotypes, with all EC_{50}'s > 10 μM. Although Byrd and co-workers(Byrd et al., 2013) found that the DENV-2 serotype was the most sensitive serotype to this capsid inhibitor and showed up to two log of variability in the inhibition against other serotypes, they did not fully evaluate the variation in susceptibility to other serotypes sufficiently comprehensively to draw conclusions. In their study, they associated ST-148 resistance to a Leucine at position 34 instead of a Serine in DENV-2. However, looking at capsid amino-acid alignment of all our DENV panel and their study's strains, and regardless of their sensibility to ST-148, all strains exhibited a Serine at position 34 except DENV-2 from Trinidad (1751 TC 544), which presented a Proline at this position, as the Modoc virus that they reported to be sensitive to ST-148 (Byrd et al., 2013). Thus, if resistance for ST-148 can arise from S34L mutations in some DENV-2 strains it is clear that it cannot be unequivocally associated to a Leucine in position 34 in other serotypes and genotypes. This suggests that other residues or domains in the capsid protein may be involved in the interaction.

SDM25N showed moderate efficacy, with EC_{50}'s ranging from 1.7 to 7.7 μM against a large proportion of the DENV-2 genotype strains, and half of the DENV-1 genotypes. However, no activity was observed against any of the DENV-3 and 4 genotypes, as EC_{50} were all above 10 μM. This result suggests that the binding affinity of NS4B to the hypothetical cellular factor targeted by SDM25N varies greatly among various DENV genotypes and/or that this cellular factor might be dispensable for efficient replication of some DENV genotypes.

Overall, the results demonstrate that compounds targeting highly conserved sites, exemplified by nucleoside analogue inhibitor NITD008 (targeting the active site of the polymerase), had a broader pan-serotypic activity, with similar EC_{50}'s regardless of the DENV genotype. In contrast, compounds targeting less conserved proteins or protein subdomains, either directly (*e.g.* the capsid) or indirectly through an interaction with a host factor of the cell (*e.g.* SDM25N), exhibited larger differences in activity towards the various genotypes of DENV.

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

Funding

This research was carried out under sponsorship of Janssen-Cilag S. A., a pharmaceutical company of Johnson & Johnson (Research agreement ICD#1041950) and EVAg European Union – Horizon 2020 program under grant agreement no. 653316; http://www.european-virusarchive.com).

Contributions

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

Acknowledgments

We would thank Dr Robert Tesh from UTMB (Texas) for providing the dengue 4 Genotype I strain G11337 from India. We thank Dr Raphaelle Klitting for her help and expertise regarding the dengue phylogeny. We would thank Pr E.A Gould and Dr Stéphane Priet for their careful reading of the manuscript. As well as Magali Gilles, and Fiona Baudino from the UMR UVE (Marseille, France) for excellent

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

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

technical help. We also thank Geraldine Piorkowski and Karine Barthelemy from UMR UVE (Marseille, France) for the sequencing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.05.005.

References

Atieh, T., Baronti, C., de Lamballerie, X., Nougairède, A., 2016. Simple reverse genetics systems for Asian and African Zika viruses. Sci. Rep. 6, 39384. https://doi.org/10.1038/srep39384.

Aubry, F., Nougairède, A., de Fabritus, L., Piorkowski, G., Gould, E.A., de Lamballerie, X., 2015. "ISA-Lation" of single-stranded positive-sense RNA viruses from non-infectious clinical/animal samples. PLoS One 10, e0138703. https://doi.org/10.1371/journal.pone.0138703.

Aubry, F., Nougairède, A., de Fabritus, L., Querat, G., Gould, E.A., de Lamballerie, X., 2014. Single-stranded positive-sense RNA viruses generated in days using infectious subgenomic amplicons. J. Gen. Virol. 95, 2462–2467. https://doi.org/10.1099/vir.0.068023-0.

Byrd, C.M., Dai, D., Grosenbach, D.W., Berhanu, A., Jones, K.F., Cardwell, K.B., Schneider, C., Wineinger, K.A., Page, J.M., Harver, C., Stavale, E., Tyavanagimatt, S., Stone, M.A., Bartenschlager, R., Scaturro, P., Hruby, D.E., Jordan, R., 2013. A novel inhibitor of dengue virus replication that targets the capsid protein. Antimicrob. Agents Chemother. 57, 15–25. https://doi.org/10.1128/AAC.01429-12.

Canard, B., 2012. Antiviral Research and Development against Dengue Virus. Available Accessed 9.

Carrillo-Valenzo, E., Danis-Lozano, R., Velasco-Hernández, J.X., Sánchez-Burgos, G., Alpuche, C., López, I., Rosales, C., Baronti, C., de Lamballerie, X., Holmes, E.C., Ramos-Castañeda, J., 2010. Evolution of dengue virus in Mexico is characterized by frequent lineage replacement. Arch. Virol. 155, 1401–1412. https://doi.org/10.1007/s00705-010-0721-1.

Chen, R., Vasilakis, N., 2011. Dengue — quo tu et quo vadis? Viruses 3, 1562–1608. https://doi.org/10.3390/v3091562.

Coutard, B., Gorbalenya, A.E., Snijder, E.J., Leontovich, A.M., Poupon, A., De Lamballerie, X., Charrel, R., Gould, E.A., Gunther, S., Norder, H., Klempa, B., Bourhy, H., Rohayem, J., L'hermite, E., Nordlund, P., Stuart, D.I., Owens, R.J., Grimes, J.M., Tucker, P.A., Bolognesi, M., Mattevi, A., Coll, M., Jones, T.A., Åqvist, J., Unge, T., Hilgenfeld, R., Bricogne, G., Neyts, J., La Colla, P., Puerstinger, G., Gonzalez, J.P., Leroy, E., Cambillau, C., Romette, J.L., Canard, B., 2008. The VIZIER project: preparedness against pathogenic RNA viruses. Antiviral Res., Special Issue: Treatment of highly pathogenic RNA viral infections 78, 37–46. https://doi.org/10.1016/j.antiviral.2007.10.013.

Delang, L., Li, C., Tas, A., Quérat, G., Albulescu, I.C., De Burghgraeve, T., Guerrero, N.A.S., Gigante, A., Piorkowski, G., Decroly, E., Jochmans, D., Canard, B., Snijder, E.J., Pérez-Pérez, M.J., van Hemert, M.J., Coutard, B., Leyssen, P., Neyts, J., 2016. The viral capping enzyme nsP1: a novel target for the inhibition of chikungunya virus infection. Sci. Rep. 6, 31819. https://doi.org/10.1038/srep31819.

Gebhard, L.G., Filomatori, C.V., Gamarnik, A.V., 2011. Functional RNA elements in the dengue virus genome. Viruses 3, 1739–1756. https://doi.org/10.3390/v3091739.

Guzman, M.G., Harris, E., 2015. Dengue. The Lancet 385, 453–465. https://doi.org/10.1016/S0140-6736(14)60572-9.

Kaptein, S.J., Neyts, J., 2016. Towards antiviral therapies for treating dengue virus infections. Curr. Opin. Pharmacol. 30, 1–7. https://doi.org/10.1016/j.coph.2016.06.002.

Katzelnick, L.C., Gresh, L., Halloran, M.E., Mercado, J.C., Kuan, G., Gordon, A., Balmaseda, A., Harris, E., 2017. Antibody-dependent enhancement of severe dengue disease in humans. Science 358, 929–932. https://doi.org/10.1126/science.aan6836.

Messina, J.P., Brady, O.J., Scott, T.W., Zou, C., Pigott, D.M., Duda, K.A., Bhatt, S., Katzelnick, L., Howes, R.E., Battle, K.E., Simmons, C.P., Hay, S.I., 2014. Global spread of dengue virus types: mapping the 70 year history. Trends Microbiol. 22, 138–146. https://doi.org/10.1016/j.tim.2013.12.011.

Romette, J.L., Prat, C.M., Gould, E.A., de Lamballerie, X., Charrel, R., Coutard, B., Fooks, A.R., Bardsley, M., Carroll, M., Drosten, C., Drexler, J.F., Günther, S., Klempa, B., Pinschewer, D., Klimkait, T., Avsic-Zupanc, T., Capobianchi, M.R., Dicaro, A., Ippolito, G., Nitsche, A., Koopmans, M., Reusken, C., Gorbalenya, A., Raoul, H., Bourhy, H., Mettenleiter, T., Reiche, S., Batten, C., Sabeta, C., Paweska, J.T., Eropkin, M., Zverev, V., Hu, Z., Mac Cullough, S., Mirazimi, A., Pradel, F., Lieutaud, P., 2018. The European Virus Archive goes global: a growing resource for research. Antivir. Res. 158. https://doi.org/10.1016/j.antiviral.2018.07.017.

van Cleef, K.W.R., Overheul, G.J., Thomassen, M.C., Kaptein, S.J.F., Davidson, A.D., Jacobs, M., Neyts, J., van Kuppeveld, F.J.M., van Rij, R.P., 2013. Identification of a new dengue virus inhibitor that targets the viral NS4B protein and restricts genomic RNA replication. Antivir. Res. 99, 165–171. https://doi.org/10.1016/j.antiviral.2013.05.011.

Vasilakis, N., Weaver, S.C., 2017. Flavivirus transmission focusing on Zika. Curr. Opin. Virol., Emerging viruses: intraspecies transmission • Viral immunology 22, 30–35. https://doi.org/10.1016/j.coviro.2016.11.007.

Vilela, A.P.P., Miranda, D.P.J., Andrade, E.H.P., Abrahão, J.S., Araújo, V.E.M., Zibaoui, H.M., Oliveira, J.G., Rosa, J.C.C., Figueiredo, L.B., Kroon, E.G., Ferreira, P.C.P., 2016. Spatial–temporal Co-circulation of dengue virus 1, 2, 3, and 4 associated with coinfection cases in a hyperendemic area of Brazil: a 4-week survey. Am. J. Trop. Med. Hyg. 94, 1080–1084. https://doi.org/10.4269/ajtmh.15-0892.

Weaver, S.C., Vasilakis, N., 2009. Molecular evolution of dengue viruses: contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. Infect. Genet. Evol. 9, 523–540. https://doi.org/10.1016/j.meegid.2009.02.003.

Xie, X., Zou, J., Wang, Q.-Y., Shi, P.-Y., 2015. Targeting dengue virus NS4B protein for drug discovery. Antivir. Res. 118, 39–45. https://doi.org/10.1016/j.antiviral.2015.03.007.

Yin, Z., Chen, Y.-L., Schul, W., Wang, Q.-Y., Gu, F., Duraiswamy, J., Kondreddi, R.R., Niyomrattanakit, P., Lakshminarayana, S.B., Goh, A., Xu, H.Y., Liu, W., Liu, B., Lim, J.Y.H., Ng, C.Y., Qing, M., Lim, C.C., Yip, A., Wang, G., Chan, W.L., Tan, H.P., Lin, K., Zhang, B., Zou, G., Bernard, K.A., Garrett, C., Beltz, K., Dong, M., Weaver, M., He, H., Pichota, A., Dartois, V., Keller, T.H., Shi, P.-Y., 2009. An adenosine nucleoside inhibitor of dengue virus. Proc. Natl. Acad. Sci. U.S.A. 106, 20435–20439. https://doi.org/10.1073/pnas.0907010106.