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# Rapid genotyping protocol to improve dengue virus serotype 2 survey in Lao PDR

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## Abstract

Dengue fever is one of the major public health problems in Lao PDR. Over the last decade, dengue virus (DENV) epidemics were characterized by a novel predominant serotype accompanied by at least two other serotypes. Since 2008, DENV-2 circulated at a low level in Lao PDR but its epidemiologic profile changed at the end of 2018. Indeed, the number of confirmed DENV-2 cases suddenly increased in October 2018 and DENV-2 became predominant at the country level in early 2019. We developed a Genotype Screening Protocol (GSP) to determine the origin(s) of the Lao DENV-2 and study their genetic polymorphism. With a good correlation with full envelope gene sequencing data, this molecular epidemiology tool evidence the co-circulation of two highly polymorphic DENV-2 genotypes, i.e. Asian I and Cosmopolitan genotypes, over the last five years, suggesting multiple introductions of DENV-2 in the country. GSP approach provides relevant first line information that may help countries with limited laboratory resources to reinforce their capabilities to DENV-2 and to follow the epidemics progresses and assess situations at the regional level.

## Introduction

Dengue fever is the most prevalent arboviral disease in the world. It represents a threat for 390 million people in 128 countries primarily in Latin America, Western Pacific and in South East Asia [1, 2]. The World Health Organization (WHO) estimated that at least 500,000 people, mostly children, are hospitalized annually; of whom 2.5% have fatal outcomes.

Lao People's Democratic Republic (PDR) is a low-middle income country located at a central position of the Indochinese peninsula. Lao PDR has a population of 6.76 million with nearly 40% under the age of 20 (sources: WHO; www.lsb.gov.la). The first dengue virus (DENV) outbreak in Lao PDR was reported in 1979 [3]. Since then, several epidemics have been recorded in the country that raise dengue to the rank of a major public health concern for their national authorities [3]. Previous studies have highlighted the dramatic increase in commercial and tourism exchanges between Lao PDR and its neighboring countries as the driving force behind the entry of multiple DENV serotypes and/or genotypes into the country

[4, 5]. The emergence of a new DENV serotype or genotype has been reported to enhance the number of cases and the diseases's severity [6–8].

In 2012, an arbovirus laboratory surveillance system was set up in the capital city of Vientiane to improve arboviral diagnosis and prevention in the country [5, 9, 10]. Since 2012, the Vientiane Capital Province has experienced two major DENV epidemics, first with a predominance of DENV-3 in 2012–2013 and then DENV-4 from the end of 2014 to end of 2018 [4, 5, 9, 11]. Over this period, DENV-2 was only the cause of 3 to 20% of confirmed cases [5] (https://www.pasteur.la/). However, in October 2018 DENV-2 burden progressively increased and reached 43% of the confirmed dengue infections in Vientiane capital the following December [12]. Since the beginning of 2019, DENV-2 has become the predominant serotype; representing 68.9% of the samples serotyped in the capital [13]. Of the 21 fatal cases recorded in 2019 in Vientiane Capital by the arbovirus surveillance system, six could be serotyped, of which four were assigned to DENV-2. At the country level, DENV-2 has been reported in 12 of the 18 provinces (Fig 1).

Little is known concerning DENV-2 genotype circulation and phylogeny in Lao PDR. Here we report on the development of a Genotype Screening Protocol (GSP) based on partial E gene sequencing for the rapid identification of DENV-2 genotyping and its origin. Performance of the GSP was compared to full E gene sequencing. GSP was also retrospectively applied to a panel of DENV-2 positive samples or isolates collected over the last eight years in Vientiane Capital as well as other Lao provinces.

## Materials and methods

### Sample collection

A panel of 53 DENV-2 isolates, screened by the arbovirus surveillance network as previously described [5], of autochthonous (51 samples) and imported cases (2 samples) across six Lao provinces between 2012 to 2019, was analyzed (Fig 1; Table 1).

### Ethic statement

The National Ethic Committee for Health Research of the Ministry of Health of Lao PDR approved the protocol used in this study. This protocol was also approved by all public hospitals' management committees and the agreement of the Ministry of health was obtained.

For all the patient samples included in this study, a written informed consent was provided by all the adult volunteers or by a parent or legal guardian on behalf of their children.

### Screening of dengue suspected cases and serotype identification

RNA was extracted from the plasma of suspected dengue cases using Nucleospin RNA virus kit (Macherey-Nagel) following the manufacturer's instructions. Sample RNA was first screened by mean of a pan-dengue virus one-step RT-PCR as described previously [5, 14]. DENV serotype identification was tentatively determined using primer/probe sets described by Ito *et al* adapted to a multiplex one-step RT-PCR format [15].

### Gene E sequencing analysis

The DENV genotypes and their geographic origin(s) were initially determined by the rapid Genotype Screening Protocol (GSP) based on partial envelope gene sequencing. Viral genome RNA was extracted from human plasma when the Cq value of the screening RT-PCR was below 30. For samples which displayed a Cq value above 30, and when the remaining volume was sufficient, DENV was isolated on C6/36 monolayers to improve the chance of sequencing.


**Fig 1. Map of Lao PDR.** Provinces with DENV-2 reported cases are in yellow. Triangles indicate the localization of DENV-2 isolates which were sequenced in this study. This map was generated by IPL staff using Inkscape software free of copyright.

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Such additional viral amplification has been documented to have no impact on viral genome stability [16]. In these cases, the above RNA extraction protocol was applied to viral culture supernatants. For sequencing purposes, first strand cDNA was generated using random hexamers and the Maxima H Minus First Strand cDNA Synthesis kit (Thermo Scientific). PCR was performed using Phusion Flash High-Fidelity PCR Master Kit (New England Biolabs® Inc). GSP (552 nt) was performed using FG4 primers (Table 2). Complete envelope gene sequences (1485 nt) were established for a panel of samples (Table 2) selected within the clusters obtained by GSP using primers FR2 or FGT2 (respectively adapted for Cosmopolitan and Asian I genotypes), FGT3 and FG4 (Table 2). Amplified fragments were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific) or by purification of PCR products from agarose gel using the Cleanup Gel extraction kit (Macherey-Nagel) when

**Table 1. References of Lao DENV-2 isolates.**

| Sample identification | Years of collection | RNA source | GenBank accession number | | Cq[(2)] |
|---|---|---|---|---|---|
| | | | GSP[(1)] | Full Envelopee protein | |
| LaoPDR-Vientiane Capital -2015-3231 | 2015 | Culture supernatant | MN444556 | MN444605 | 27 |
| LaoPDR-Vientiane Capital -2016-3550 | 2016 | Culture supernatant | MN444557 | MN444606 | 24 |
| LaoPDR-Vientiane Capital -2016-3771 | 2016 | Culture supernatant | MN444558 | MN444607 | 26 |
| LaoPDR-Vientiane Capital -2016-3831 | 2016 | Plasma | MN444559 | MN444608 | 25 |
| LaoPDR-Vientiane Capital -2017-5207 | 2017 | Culture supernatant | MN444560 | MN444609 | 20 |
| LaoPDR-Vientiane Capital -2017-5208 | 2017 | Culture supernatant | MN444561 | MN444610 | 21 |
| LaoPDR-Vientiane Capital -2017-5443 | 2017 | Culture supernatant | MN444562 | MN444611 | 24 |
| LaoPDR-Vientiane Capital -2017-5902 | 2017 | Plasma | MN444563 | F | 20 |
| LaoPDR-Vientiane Capital -2017-5955 | 2017 | Culture supernatant | MN444564 | F | 25 |
| LaoPDR-Vientiane Capital -2017-6086 | 2017 | Plasma | MN444565 | F | 21 |
| LaoPDR-Vientiane Capital -2017-6177 | 2017 | Plasma | MN444566 | F | 25 |
| LaoPDR-Vientiane Capital -2017-6446 | 2017 | Culture supernatant | MN444567 | MN444612 | 22 |
| LaoPDR-Vientiane Capital -2018-7406 | 2018 | Culture supernatant | MN444568 | MN444613 | 22 |
| LaoPDR-Vientiane Capital -2018-7692 | 2018 | Plasma | MN444569 | ND | 31 |
| LaoPDR-Vientiane Capital -2018-7740 | 2018 | Plasma | MN444570 | F | 22 |
| LaoPDR-Vientiane Capital -2018-7800 | 2018 | Plasma | MN444571 | ND | 23 |
| LaoPDR-Vientiane Capital -2018-7944 | 2018 | Plasma | MN444572 | F | 23 |
| LaoPDR-Vientiane Capital -2018-8075 | 2018 | Plasma | MN444573 | ND | 21 |
| LaoPDR-Vientiane Capital -2018-8173 | 2018 | Plasma | MN444574 | MN444614 | 22 |
| LaoPDR-Vientiane Capital -2018-8359 | 2018 | Plasma | MN444575 | ND | 25 |
| LaoPDR-Vientiane Capital -2018-8366 | 2018 | Plasma | MN444576 | ND | 26 |
| LaoPDR-Vientiane Capital (fatal case)-2018-8372 | 2018 | Culture supernatant | MN444577 | MN444615 | 18 |
| LaoPDR-Vientiane Capital -2018-8509 | 2018 | Plasma | MN444578 | ND | 29 |
| LaoPDR-Vientiane Capital -2018-8518 | 2018 | Plasma | MN444579 | ND | 24 |
| LaoPDR-Vientiane Capital -2018-8541 | 2018 | Plasma | MN444580 | ND | 31 |
| LaoPDR-Vientiane Capital (fatal case)-2018-8920 | 2018 | Plasma | MN444581 | MN444616 | 23 |
| LaoPDR-Vientiane Capital (fatal case)-2019-9060 | 2019 | Plasma | MN444582 | F | 36 |
| LaoPDR-Vientiane Capital (fatal case)-2019-9080 | 2019 | Culture supernatant | MN444583 | MN444617 | 18 |
| LaoPDR-Vientiane Capital -2019-9128 | 2019 | Plasma | MN444584 | ND | 20 |
| LaoPDR-Vientiane Province-2017-5545 | 2017 | Plasma | MN444585 | F | 29 |
| LaoPDR-Vientiane Province-2017-5725 | 2017 | Plasma | MN444586 | F | 25 |
| LaoPDR-Vientiane Province-2017-5942 | 2017 | Culture supernatant | MN444587 | ND | 24 |
| LaoPDR-Luangprabang-2017-5705 | 2017 | Plasma | MN444588 | MN444618 | 24 |
| LaoPDR-Luangprabang-2018-8737 | 2018 | Plasma | MN444589 | F | 26 |
| LaoPDR-Luangprabang-2018-8741 | 2018 | Plasma | MN444590 | F | 25 |
| LaoPDR-Savannakhet-2018-8014 | 2018 | Plasma | MN444591 | MN444619 | 25 |
| LaoPDR-Savannakhet-2018-8135 | 2018 | Plasma | MN444592 | ND | 20 |
| LaoPDR-Savannakhet-2018-8358 | 2018 | Plasma | MN444593 | F | 29 |
| LaoPDR-Saravane-2018-7670 | 2018 | Plasma | MN444594 | F | 26 |
| LaoPDR-Saravane-2018-7936 | 2018 | Plasma | MN444595 | F | 21 |
| LaoPDR-Saravane-2019-9444 | 2019 | Plasma | MN444596 | ND | 25 |
| LaoPDR-Saravane-2019-9474 | 2019 | Plasma | MN444597 | ND | 21 |
| LaoPDR-Attapeu-2017-5601 | 2017 | Culture supernatant | MN444598 | MN444620 | 23 |
| LaoPDR-Attapeu-2017-6144 | 2017 | Plasma | MN444599 | F | 31 |
| LaoPDR-Attapeu-2018-7507 | 2018 | Plasma | MN444600 | F | 23 |
| LaoPDR-Attapeu-2018-7511 | 2018 | Plasma | MN444601 | MN444621 | 24 |

(*Continued*)

**Table 1.** (Continued)

| Sample identification | Years of collection | RNA source | GenBank accession number | | Cq[(2)] |
|---|---|---|---|---|---|
| | | | GSP[(1)] | Full Envelopee protein | |
| LaoPDR-Attapeu-2019-9494 | 2019 | Plasma | MN444602 | ND | 22 |
| **LaoPDR-Vientiane Capital (ex Thailand)-2017-5642** | **2017** | **Plasma** | **MN444603** | **MN444622** | **27** |
| **LaoPDR-Vientiane Capital (ex Vietnam)-2018-8746** | **2018** | **Plasma** | **MN444604** | **F** | **30** |

[(1)] Genotype Screening Protocol.

[(2)] Cq were obtained from plasma or culture supernatant with pan-dengue RT-PCR from Warrilow *et al* [14]. ND: not done. F: Failed. Imported cases are indicated in bold.

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nonspecific amplification was observed by agarose gel electrophoresis. Forward and reverse strands were independently sequenced using BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystem) and loaded on a Genetic Analyzer 3500xL (Applied Biosystem).

### Phylogenetic analysis

Partial (53 sequences; GenBank MN444556 to MN444604) and complete (22 sequences; GenBank MN444605 to MN444622) envelope sequences of Lao isolates (Table 1) were compared with envelope gene sequences retrieved from GenBank (S1 Table) [4, 17–44]. Maximum-likelihood trees were constructed using MEGA version 7 (www.megasoftware.net) as previously described [5, 6, 45–47], with the Kimura-2-parameter method and a bootstrap of 1000 replications [48].

## Results

### Co-circulation of two DENV-2 genotypes in Lao PDR

Of this panel of 53 isolates, GSP verified the co-circulation of two DENV-2 genotypes. Asian I and Cosmopolitan genotypes were both detected in different Lao PDR provinces (Fig 2). The co-circulation of these genotypes was detected in 2017, 2018 and 2019 in the country.

### Persistence of DENV-2 Asian I genotype in Lao PDR

Lao DENV-2 samples isolated between 2008 and 2010 have been assigned to the Asian I genotype [4]. In our series, 19 isolates (36%) obtained after 2012 shared strong identity (>97.2%) with this genotype and demonstrates its persistence in the country for over eleven years. Lao Asian I isolates since 2008 were classed into seven clusters, displaying distances of 1.0 to 2.8% from each other at the nucleotide level (Fig 2). Three of these clusters were described

**Table 2. List and positions of primers used for partial or complete envelope gene RT-PCR and sequencing.**

| Fragment | Forward | Genome position | Reverse | Genome position |
|---|---|---|---|---|
| FR2[a] | 5′TGT-CAT-CAG-AAG-GGG-CCT-G3′ | 770–788 | 5′TCA-TTG-AAG-TCR-AGG-CCC-G3′ | 1502–1520 |
| FGT2 [a] | 5′TCA-CCA-TAA-TGG-CAG-CAA-TC3′ | 836–855 | 5′TGC-ACC-AGC-CAA-GCT-TTA-TT3′ | 1543–1562 |
| FGT3 [a] | 5′ACA-CCA-TTG-TGR-TAA-CAC-C3′ | 1346–1364 | 5′TCT-GCT-TCT-ATG-TTG-ACT-G3′ | 2027–2045 |
| FG4 [a, b] | 5′TGT-GAA-GGA-AAT-AGC- AGA 3′ | 1860–1877 | 5′AGT-TCT-TTG-TTT-TTC-CAG-CT 3′ | 2440–2459 |

The genome positions are given according to the dengue virus serotype 2 reference genome (GenBank: U87411).

[a] indicates the primers used for complete envelope gene sequencing and

[b] for the Genotype Screening Protocol.

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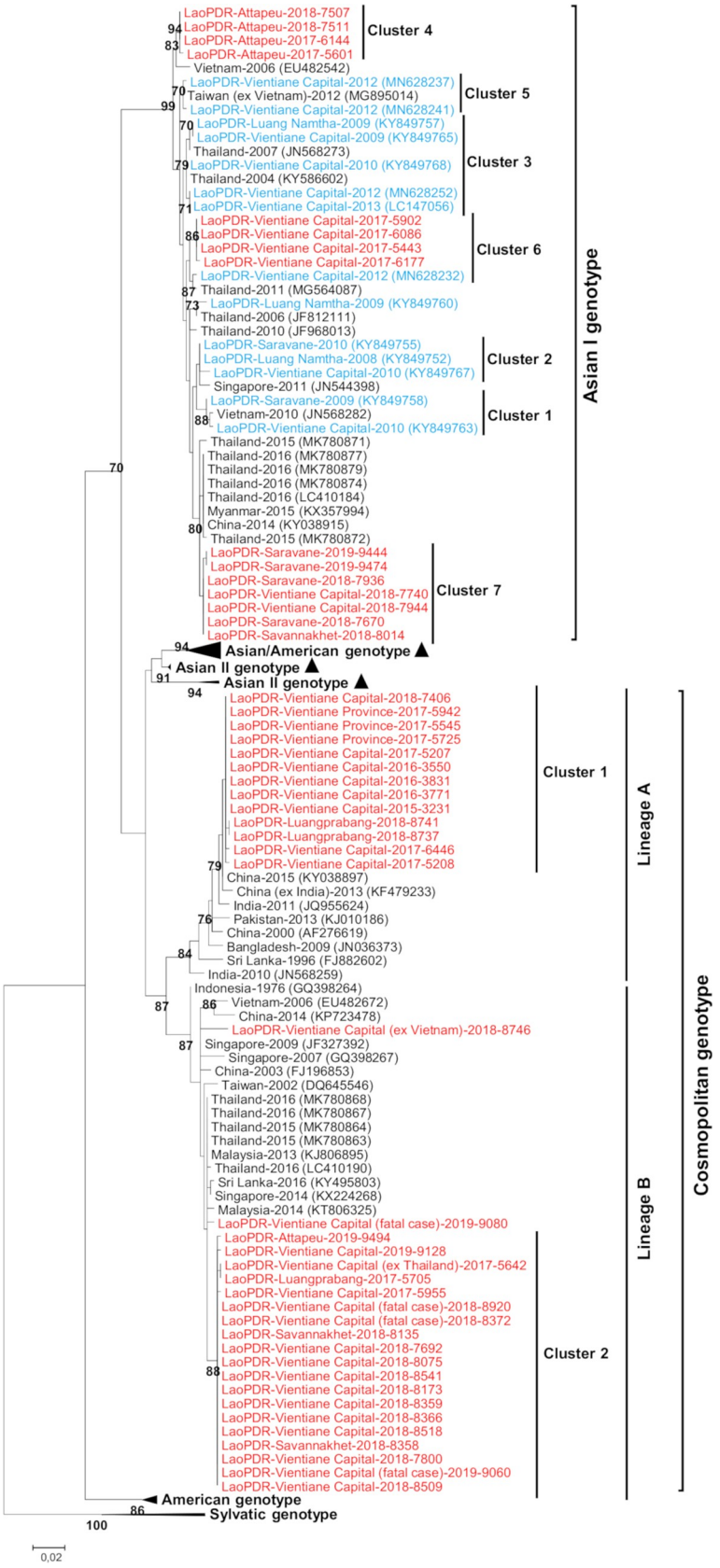
LaoPDR-Attapeu-2018-7507
LaoPDR-Attapeu-2018-7511
LaoPDR-Attapeu-2017-6144
LaoPDR-Attapeu-2017-5601
Cluster 4
Vietnam-2006 (EU482542)
LaoPDR-Vientiane Capital-2012 (MN628237)
Taiwan (ex Vietnam)-2012 (MG895014)
LaoPDR-Vientiane Capital-2012 (MN628241)
Cluster 5
LaoPDR-Luang Namtha-2009 (KY849757)
LaoPDR-Vientiane Capital-2009 (KY849765)
Thailand-2007 (JN568273)
LaoPDR-Vientiane Capital-2010 (KY849768)
Thailand-2004 (KY586602)
LaoPDR-Vientiane Capital-2012 (MN628252)
LaoPDR-Vientiane Capital-2013 (LC147056)
Cluster 3
LaoPDR-Vientiane Capital-2017-5902
LaoPDR-Vientiane Capital-2017-6086
LaoPDR-Vientiane Capital-2017-5443
LaoPDR-Vientiane Capital-2017-6177
LaoPDR-Vientiane Capital-2012 (MN628232)
Cluster 6
Thailand-2011 (MG564087)
LaoPDR-Luang Namtha-2009 (KY849760)
Thailand-2006 (JF812111)
Thailand-2010 (JF968013)
LaoPDR-Saravane-2010 (KY849755)
LaoPDR-Luang Namtha-2008 (KY849752)
LaoPDR-Vientiane Capital-2010 (KY849767)
Cluster 2
Singapore-2011 (JN544398)
LaoPDR-Saravane-2009 (KY849758)
Vietnam-2010 (JN568282)
LaoPDR-Vientiane Capital-2010 (KY849763)
Cluster 1
Asian I genotype
Thailand-2015 (MK780871)
Thailand-2016 (MK780877)
Thailand-2016 (MK780879)
Thailand-2016 (MK780874)
Thailand-2016 (LC410184)
Myanmar-2015 (KX357994)
China-2014 (KY038915)
Thailand-2015 (MK780872)
LaoPDR-Saravane-2019-9444
LaoPDR-Saravane-2019-9474
LaoPDR-Saravane-2018-7936
LaoPDR-Vientiane Capital-2018-7740
LaoPDR-Vientiane Capital-2018-7944
LaoPDR-Saravane-2018-7670
LaoPDR-Savannakhet-2018-8014
Cluster 7
Asian/American genotype
Asian II genotype
Asian II genotype
LaoPDR-Vientiane Capital-2018-7406
LaoPDR-Vientiane Province-2017-5942
LaoPDR-Vientiane Province-2017-5545
LaoPDR-Vientiane Province-2017-5725
LaoPDR-Vientiane Capital-2017-5207
LaoPDR-Vientiane Capital-2016-3550
LaoPDR-Vientiane Capital-2016-3831
LaoPDR-Vientiane Capital-2016-3771
LaoPDR-Vientiane Capital-2015-3231
LaoPDR-Luangprabang-2018-8741
LaoPDR-Luangprabang-2018-8737
LaoPDR-Vientiane Capital-2017-6446
LaoPDR-Vientiane Capital-2017-5208
Cluster 1
Lineage A
China-2015 (KY038897)
China (ex India)-2013 (KF479233)
India-2011 (JQ955624)
Pakistan-2013 (KJ010186)
China-2000 (AF276619)
Bangladesh-2009 (JN036373)
Sri Lanka-1996 (FJ882602)
India-2010 (JN568259)
Indonesia-1976 (GQ398264)
Vietnam-2006 (EU482672)
China-2014 (KP723478)
LaoPDR-Vientiane Capital (ex Vietnam)-2018-8746
Singapore-2009 (JF327392)
Singapore-2007 (GQ398267)
China-2003 (FJ196853)
Taiwan-2002 (DQ645546)
Thailand-2016 (MK780868)
Thailand-2016 (MK780867)
Thailand-2015 (MK780864)
Thailand-2015 (MK780863)
Malaysia-2013 (KJ806895)
Thailand-2016 (LC410190)
Sri Lanka-2016 (KY495803)
Singapore-2014 (KX224268)
Malaysia-2014 (KT806325)
LaoPDR-Vientiane Capital (fatal case)-2019-9080
LaoPDR-Attapeu-2019-9494
LaoPDR-Vientiane Capital-2019-9128
LaoPDR-Vientiane Capital (ex Thailand)-2017-5642
LaoPDR-Luangprabang-2017-5705
LaoPDR-Vientiane Capital-2017-5955
LaoPDR-Vientiane Capital (fatal case)-2018-8920
LaoPDR-Vientiane Capital (fatal case)-2018-8372
LaoPDR-Savannakhet-2018-8135
LaoPDR-Vientiane Capital-2018-7692
LaoPDR-Vientiane Capital-2018-8075
LaoPDR-Vientiane Capital-2018-8541
LaoPDR-Vientiane Capital-2018-8173
LaoPDR-Vientiane Capital-2018-8359
LaoPDR-Vientiane Capital-2018-8366
LaoPDR-Vientiane Capital-2018-8518
LaoPDR-Savannakhet-2018-8358
LaoPDR-Vientiane Capital-2018-7800
LaoPDR-Vientiane Capital (fatal case)-2019-9060
LaoPDR-Vientiane Capital-2018-8509
Cluster 2
Lineage B
Cosmopolitan genotype
American genotype
Sylvatic genotype
0,02

**Fig 2. Maximum-likelihood phylogenetic tree of DENV-2 sequences from Lao PDR.** The tree was constructed on a 552nt segment of a partial envelope protein gene (Genotype Screening Protocol). Bootstrap values >70 are shown next the node. The Lao PDR strains analyzed in this study are indicated in red. The Lao PDR strains previously described are in blue. Cluster 1–3 were previously described by Castonguay-Vanier *et al*). Scale bar indicates nucleotide substitution per site. The triangles indicate discordance between Genotype Screening Protocol and full envelope protein gene sequencing at the genotype and lineage level.

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previously and one was found specifically from the Attapeu province [4]. All the Lao isolates showed strong links with strains from Vietnam, Thailand, China, Taiwan and Myanmar.

## Co-circulation of different lineages and clusters within DENV-2 cosmopolitan genotype

A total of 34 isolates (64%) belonged to the Cosmopolitan genotype, and analysis showed that it may has been circulating in Lao PDR since at least 2015. Interestingly, the Cosmopolitan Lao isolates split into two distinct lineages (A; B), displaying 4.9–5.7% divergence from each other (bootstrap 87%). These lineages have coexisted at least as early as 2017. Within Lineage A, all Lao isolates grouped together sharing more than 99.45% identity (Cluster 1). Strong links (>98.7% identity; bootstrap 79%) were found between Lineage A with DENV-2 strains circulating in China and India in 2015 and 2011/2013 respectively. All four fatal cases included in this series belonged to Lineage B (Fig 2). Three of the four fatal cases grouped with the rest of the autochthonous isolates in a major cluster (Cluster 2, Fig 2). Surprisingly, the last fatal case (LaoPDR-Vientiane Capital-2019-9080) displayed between 1.1 and 2.1% nucleotide distance with the rest of Cluster 2. Autochthonous Lao isolates belonging to Lineage B strongly linked with DENV-2 strains from Thailand/Malaysia/Singapore/Sri-Lanka. One of the two imported cases (ex Thailand), fell in Cluster 2 whereas the second (ex Vietnam) grouped with other regional isolates detected in China, India, Bangladesh, Sri Lanka, and Southeast Asia.

## Validation of a Genotype Screening Protocol

To assess the accuracy of the GSP, the genotype/lineage/cluster assignments of 8 Asian I and 14 Cosmopolitan isolates were checked by full length envelope gene sequencing (Fig 3). The distribution of reference strains and Lao isolates established by the full envelope genotyping globally matched with the GSP results including at the levels of lineages and/or clusters. However, differences in the topology of the trees were observed for the Asian II genotype, for which some strains were previously found more closely related to the Asian/American genotype by GSP. At a higher level, the Asian II and the Asian/American genotypes were linked to the Cosmopolitan genotype by GSP rather than to the Asian I genotype as with full envelope sequencing (Fig 3).

In the subset selected for the complete envelope gene sequencing, sequences could not be established for some samples (plasma or culture supernatants) with a Cq>30 when tested with the screening real time RT-PCR (Table 1). For instance, from the 4 investigated fatal DENV-2 cases, the genotype of one sample (LaoPDR-Vientiane Capital-2019-9060) with a Cq = 36 could only be determined by GSP whereas the full envelope gene sequencing failed.

# Discussion

Dengue molecular epidemiology in Lao PDR is complex and the dynamic over time depends in part on virus serotypes and geographic origins [4, 5]. Indeed, Lao PDR is localized in a hyperendemic DENV region in Southeast Asia in the middle of the Indochinese peninsula [49]. Since the first dengue outbreak in 1979, the four DENV serotypes are frequently detected

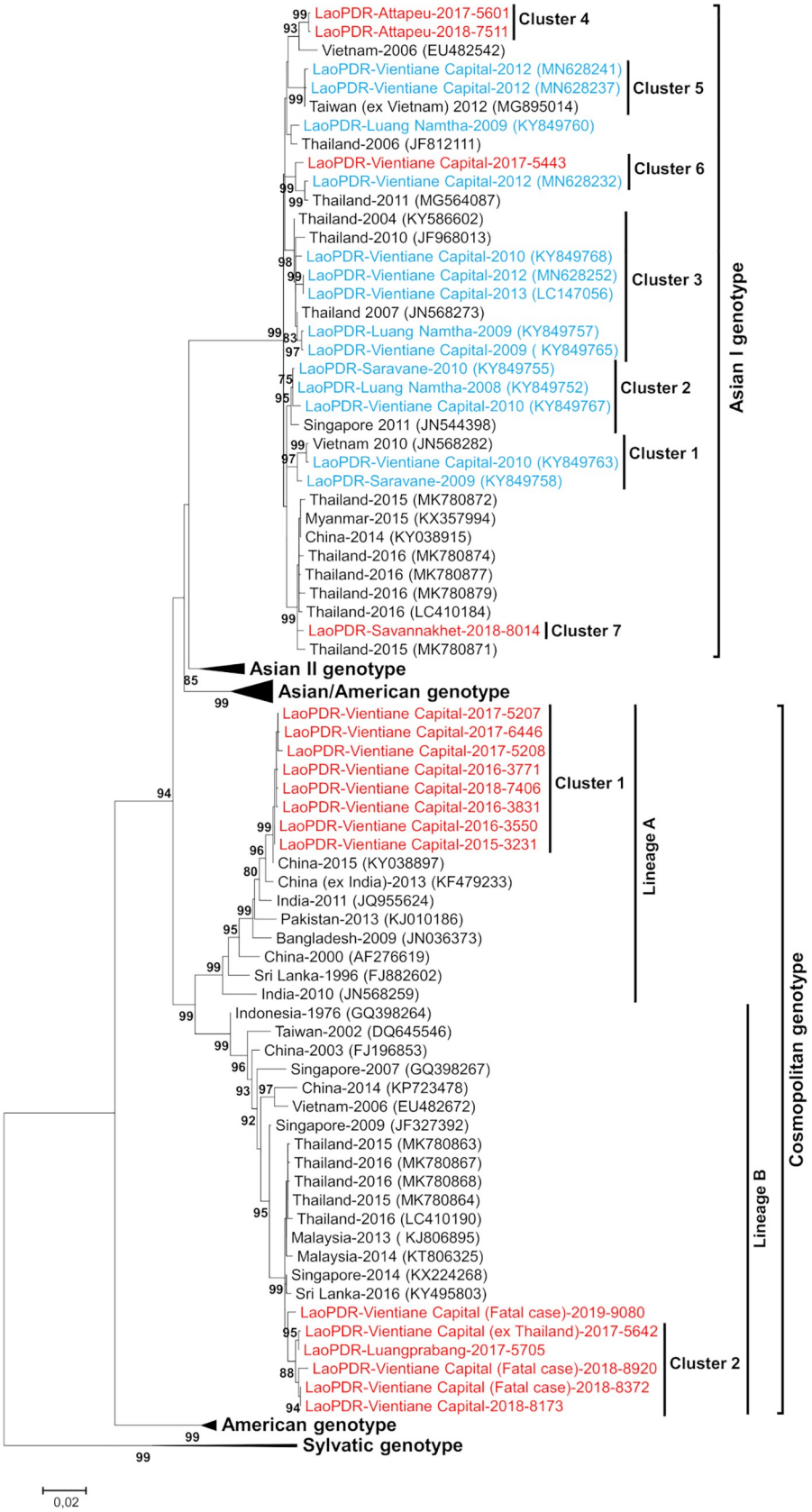
LaoPDR-Attapeu-2017-5601
LaoPDR-Attapeu-2018-7511
Cluster 4
Vietnam-2006 (EU482542)
LaoPDR-Vientiane Capital-2012 (MN628241)
LaoPDR-Vientiane Capital-2012 (MN628237)
Cluster 5
Taiwan (ex Vietnam) 2012 (MG895014)
LaoPDR-Luang Namtha-2009 (KY849760)
Thailand-2006 (JF812111)
LaoPDR-Vientiane Capital-2017-5443
LaoPDR-Vientiane Capital-2012 (MN628232)
Cluster 6
Thailand-2011 (MG564087)
Thailand-2004 (KY586602)
Thailand-2010 (JF968013)
LaoPDR-Vientiane Capital-2010 (KY849768)
LaoPDR-Vientiane Capital-2012 (MN628252)
LaoPDR-Vientiane Capital-2013 (LC147056)
Cluster 3
Thailand 2007 (JN568273)
LaoPDR-Luang Namtha-2009 (KY849757)
LaoPDR-Vientiane Capital-2009 ( KY849765)
LaoPDR-Saravane-2010 (KY849755)
LaoPDR-Luang Namtha-2008 (KY849752)
LaoPDR-Vientiane Capital-2010 (KY849767)
Cluster 2
Singapore 2011 (JN544398)
Vietnam 2010 (JN568282)
LaoPDR-Vientiane Capital-2010 (KY849763)
LaoPDR-Saravane-2009 (KY849758)
Cluster 1
Asian I genotype
Thailand-2015 (MK780872)
Myanmar-2015 (KX357994)
China-2014 (KY038915)
Thailand-2016 (MK780874)
Thailand-2016 (MK780877)
Thailand-2016 (MK780879)
Thailand-2016 (LC410184)
LaoPDR-Savannakhet-2018-8014
Cluster 7
Thailand-2015 (MK780871)
Asian II genotype
Asian/American genotype
LaoPDR-Vientiane Capital-2017-5207
LaoPDR-Vientiane Capital-2017-6446
LaoPDR-Vientiane Capital-2017-5208
LaoPDR-Vientiane Capital-2016-3771
LaoPDR-Vientiane Capital-2018-7406
LaoPDR-Vientiane Capital-2016-3831
LaoPDR-Vientiane Capital-2016-3550
LaoPDR-Vientiane Capital-2015-3231
Cluster 1
China-2015 (KY038897)
China (ex India)-2013 (KF479233)
India-2011 (JQ955624)
Pakistan-2013 (KJ010186)
Bangladesh-2009 (JN036373)
China-2000 (AF276619)
Sri Lanka-1996 (FJ882602)
India-2010 (JN568259)
Lineage A
Indonesia-1976 (GQ398264)
Taiwan-2002 (DQ645546)
China-2003 (FJ196853)
Singapore-2007 (GQ398267)
China-2014 (KP723478)
Vietnam-2006 (EU482672)
Singapore-2009 (JF327392)
Thailand-2015 (MK780863)
Thailand-2016 (MK780867)
Thailand-2016 (MK780868)
Thailand-2015 (MK780864)
Thailand-2016 (LC410190)
Malaysia-2013 ( KJ806895)
Malaysia-2014 (KT806325)
Singapore-2014 (KX224268)
Sri Lanka-2016 (KY495803)
LaoPDR-Vientiane Capital (Fatal case)-2019-9080
LaoPDR-Vientiane Capital (ex Thailand)-2017-5642
LaoPDR-Luangprabang-2017-5705
LaoPDR-Vientiane Capital (Fatal case)-2018-8920
LaoPDR-Vientiane Capital (Fatal case)-2018-8372
LaoPDR-Vientiane Capital-2018-8173
Cluster 2
Lineage B
Cosmopolitan genotype
American genotype
Sylvatic genotype
0,02

**Fig 3. Maximum-likelihood phylogenetic tree of DENV-2 sequences from Lao PDR.** The tree was constructed on a 1485nt segment of the envelope protein gene. Only the bootstrap values >70 are shown. Scale bar indicates nucleotide substitution per site. The Lao PDR strains analyzed in this study are indicated in red. The Lao PDR strains previously described are in blue. Cluster 1–3 were previously described by Castonguay-Vanier *et al*).

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in the country [3–5, 9, 11]. DENV genotype co-circulation has already been depicted in Lao PDR for DENV-3 [5]. For DENV-2, the co-circulation of Asian I and Asian/American or Cosmopolitan genotypes has been documented in Thailand, Cambodia or Vietnam [43, 50].

In most of these situations, the genotypes coexisted over several years [50]. In Lao PDR, Asian I and Cosmopolitan genotypes have been co-circulating since at least 2017 and was still ongoing in 2019. Compared to a recent study in Thailand, our series identified different lineages within the DENV-2 Cosmopolitan genotype [43]. Indeed, in Thailand only the Cosmopolitan genotype/Lineage B was observed between 2015 and 2016. As seen elsewhere, since 2008 the Lao clusters within the Asian I genotype have had different geographic origins and evolved over time as independent topotypes as is seen on other countries [4, 43, 50]. In a panel tested previously and in this study, the co-circulation of two or three clusters were observed in 2009–2010 (cluster 1, 2 and 3), in 2012 (clusters 3 and 5), in 2017 (clusters 4 and 6) and in 2018 (clusters 4 and 7). Interestingly, some clusters seemed to be localized only in some part of Lao PDR such as clusters 2 and 6 in Vientiane, and cluster 4 in Attapeu. Though, this observation could be due to the limited size of the series. However, the close relation between cluster 4 isolates and Vietnamese prototype strains supports another hypothesis. It can be assumed that this cluster originates from a direct introduction from Vietnam as the Eastern limit of the province materialize part of the Lao-Vietnam border. A main road, rubber plantations held by Vietnamese companies, are factors that may at least in part facilitate the traffic of DENV virus in southern Lao PDR. Further investigation is needed to better document the cluster-specific circulation of DENV-2 Asian I genotype in Lao PDR and to determine factors which could impact the selection of specific DENV strains. For instance, it has already been demonstrated that specific adaptations between the vector population and DENV may lead to an enhancement of mosquitos' ability to transmit DENV [51, 52].

GSP results showed independent introduction events of DENV-2 between 2012 and 2019 in Lao PDR. Genetic links and the geographic origin of the imported cases suggest a possible role of human regional mobility as a factor of DENV-2 polymorphism through the introduction of novel genotype, lineage, or clusters into the country. Observations of the presence of the Cosmopolitan genotype with the co-circulation of two different lineages detected as early as 2016, along with the circulation of several Asian I genotype clusters since 2008 support this hypothesis. Lao PDR shares a border with five countries where DENV circulation is highly active [1, 43, 49]. Exchanges between Lao PDR and its neighboring countries is increasing constantly especially with regards to commerce [4, 5]. In this context, the identification of potential DENV introduction routes into Lao PDR and their impact on dengue epidemiology in the country will be challenging in the future.

Here, GSP demonstrated rapid preliminary information on DENV-2 polymorphisms that were in most cases confirmed by full length envelope sequencing. Tree topology differences could be due to the limited number of nucleotides used for GSP (552nt *versus* 1,485nt for full length envelope). However, GSP analytic sensitivity seemed to be higher compared to full length envelope sequencing. In this study, samples with high Cq (above 30) could be only sequenced using GSP, which can help the investigation of samples with volume limitations or for whom viral culture is not possible.

Identification of DENV serotype(s)/genotype(s) during an outbreak can help improve dengue prevention, especially in endemic countries where the disease severity is still challenging

[53]. Improvement of rapid RT-PCR diagnostic tool already help the identification of DENV serotype [5, 15].

As previously described, DENV genotype switch or co-circulation could impact DENV emergence and spread as well as modify its epidemiology profile by increasing the number of infected patients and fatal cases [6–8]. DENV genotypes may display differences in their fitness and virulence. Some studies already suggested a possible link between a sudden switch of DENV genotypes and an increase of disease severity for DENV-1, DENV-2 and DENV-3 in Asia or South America [54–57]. In our series, the four DENV-2 isolates from fatal cases in 2018 and 2019 belonged to the Cosmopolitan genotype/Lineage B which was detected in 2017, but has not had an increase in cases by the end of 2018. Parallel studies are needed to investigate the respective roles of virus pathogenicity and indirect factors such as socio-economic factors governing access to care to determine the real impact of the emergence of DENV genotypes in endemic countries like in Lao PDR [58].

In Lao PDR, herd immunity against the different dengue virus serotypes remains cryptic and it can be assumed to be low for DENV-2, as seen by the background circulation and the various introductions seen throughout this study period.

Virologic investigations are crucial for DENV epidemic prevention and follow up. Direct diagnosis by RT-PCR is at present the gold standard for confirming dengue virus infection during the acute phase of the disease. When the geographic coverage is sufficient across the country, the serotype determination by real time RT-PCR can be used to estimate the proportions of each DENV serotype and generate data that could be used as a proxy to estimate the herd immunity against each. Molecular epidemiology by partial or complete envelope gene sequencing has been used for decades to better understand DENV circulation at regional or inter-continental levels. Genotype determination reinforces the capacity to identify trans-border routes of circulation and establish a more precise timepoint of emergence of a specific cluster of DENV isolates. From that perspective, GSP is a valuable frontline molecular epidemiological tool for countries with limited laboratory resources that are confronted with dengue. Moreover, GSP could be useful to follow the circulation of DENV-2 on both a country and regional scale.

## Supporting information

**S1 Table. References of DENV-2 envelope gene sequences from GenBank used in this study.**
(DOCX)

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## References

1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013; 496(7446):504–7. https://doi.org/10.1038/nature12060 PMID: 23563266
2. Brady OJ, Gething PW, Bhatt S, Messina JP, Brownstein JS, Hoen AG, et al. Refining the global spatial limits of dengue virus transmission by evidence-based consensus. PLoS Negl Trop Dis. 2012; 6(8): e1760. https://doi.org/10.1371/journal.pntd.0001760 PMID: 22880140
3. Fukunaga T, Phommasack B, Bounlu K, Saito M, Tadano M, Makino Y, et al. Epidemiological situation of dengue infection in Lao P.D.R. Trop Med. 1994; 35(4):219–27.
4. Castonguay-Vanier J, Klitting R, Sengvilaipaseuth O, Piorkowski G, Baronti C, Sibounheuang B, et al. Molecular epidemiology of dengue viruses in three provinces of Lao PDR, 2006–2010. Aguilar PV, editor. PLoS Negl Trop Dis. 2018 Jan 29; 12(1):e0006203. https://doi.org/10.1371/journal.pntd.0006203 PMID: 29377886
5. Lao M, Caro V, Thiberges J-M, Bounmany P, Vongpayloth K, Buchy P, et al. Co-Circulation of Dengue Virus Type 3 Genotypes in Vientiane Capital, Lao PDR. Coffey LL, editor. PLoS ONE. 2014 Dec 31; 9 (12):e115569.
6. Dupont-Rouzeyrol M, Aubry M, O'Connor O, Roche C, Gourinat AC, Guigon A, et al. Epidemiological and molecular features of dengue virus type-1 in New Caledonia, South Pacific, 2001–2013. Virol J. 2014; 11:61. https://doi.org/10.1186/1743-422X-11-61 PMID: 24684835
7. Ahamed SF, Rosario V, Britto C, Dias M, Nayak K, Chandele A, et al. Emergence of new genotypes and lineages of dengue viruses during the 2012–15 epidemics in southern India. Int J Infect Dis. 2019 Jul; 84:S34–43.
8. Jiang L, Ma D, Ye C, Li L, Li X, Yang J, et al. Molecular Characterization of Dengue Virus Serotype 2 Cosmospolitan Genotype From 2015 Dengue Outbreak in Yunnan, China. Front Cell Infect Microbiol. 2018 Jun 27; 8.
9. Khampapongpane B, Lewis HC, Ketmayoon P, Phonekeo D, Somoulay V, Khamsing A, et al. National dengue surveillance in the Lao People's Democratic Republic, 2006–2012: epidemiological and laboratory findings. West Pac Surveill Response J WPSAR. 2014; 5(1):7–13.
10. Somlor S, Vongpayloth K, Diancourt L, Buchy P, Duong V, Phonekeo D, et al. Chikungunya virus emergence in the Lao PDR, 2012–2013. LFP, editor. PLOS ONE. 2017 Dec 28; 12(12):e0189879. https://doi.org/10.1371/journal.pone.0189879 PMID: 29284012
11. Soukaloun D. Dengue infection in Lao PDR. Southeast Asian J Trop Med Public Health. 2014; 45 Suppl 1:113–9.
12. Institut Pasteur du Laos. Institut Pasteur du Laos Activities Report 2018–2019. 2019.
13. Institut Pasteur du Laos. Institut Pasteur du Laos Activities Report 2019–2020. 2020.
14. Warrilow D, Northill JA, Pyke A, Smith GA. Single rapid TaqMan fluorogenic probe based PCR assay that detects all four dengue serotypes. J Med Entomol. 2002; 66(4):524–8.
15. Ito M, Takasaki T, Yamada K-I, Nerome R, Tajima S, Kurane I. Development and Evaluation of Fluorogenic TaqMan Reverse Transcriptase PCR Assays for Detection of Dengue Virus Types 1 to 4. J Clin Microbiol. 2004 Dec 1; 42(12):5935–7. https://doi.org/10.1128/JCM.42.12.5935-5937.2004 PMID: 15583346
16. Chen W-J, Wu H-R, Chiou S-S. E/NS1 Modifications of Dengue 2 Virus after Serial Passages in Mammalian and/or Mosquito Cells. Intervirology. 2003; 46(5):289–95. https://doi.org/10.1159/000073208 PMID: 14555849
17. Steel A, Gubler DJ, Bennett SN. Natural attenuation of dengue virus type-2 after a series of island outbreaks: A retrospective phylogenetic study of events in the South Pacific three decades ago. Virology. 2010 Sep; 405(2):505–12. https://doi.org/10.1016/j.virol.2010.05.033 PMID: 20663532

18. Leitmeyer KC, Vaughn DW, Watts DM, Salas R, Villalobos I, de Chacon null, et al. Dengue virus structural differences that correlate with pathogenesis. J Virol. 1999 Jun; 73(6):4738–47. https://doi.org/10.1128/JVI.73.6.4738-4747.1999 PMID: 10233934

19. Vasilakis N, Fokam EB, Hanson CT, Weinberg E, Sall AA, Whitehead SS, et al. Genetic and phenotypic characterization of sylvatic dengue virus type 2 strains. Virology. 2008 Aug; 377(2):296–307. https://doi.org/10.1016/j.virol.2008.04.044 PMID: 18570968

20. Phommanivong V, Kanda S, Shimono T, Lamaningao P, Darcy AW, Mishima N, et al. Co-circulation of the dengue with chikungunya virus during the 2013 outbreak in the southern part of Lao PDR. Trop Med Health. 2016 Dec; 44(1).

21. Kyaw AK, Ngwe Tun MM, Moi ML, Nabeshima T, Soe KT, Thwe SM, et al. Clinical, virological and epidemiological characterization of dengue outbreak in Myanmar, 2015. Epidemiol Infect. 2017 Jul; 145 (9):1886–97. https://doi.org/10.1017/S0950268817000735 PMID: 28414004

22. Warrilow D, Northill JA, Pyke AT. Sources of Dengue Viruses Imported into Queensland, Australia, 2002–2010. Emerg Infect Dis. 2012 Nov; 18(11):1850–7. https://doi.org/10.3201/eid1811.120014 PMID: 23092682

23. Yang C-F, Chang S-F, Hsu T-C, Su C-L, Wang T-C, Lin S-H, et al. Molecular characterization and phylogenetic analysis of dengue viruses imported into Taiwan during 2011–2016. Blacksell SD, editor. PLoS Negl Trop Dis. 2018 Sep 20; 12(9):e0006773. https://doi.org/10.1371/journal.pntd.0006773 PMID: 30235208

24. Vasilakis N, Holmes EC, Fokam EB, Faye O, Diallo M, Sall AA, et al. Evolutionary Processes among Sylvatic Dengue Type 2 Viruses. J Virol. 2007 Sep 1; 81(17):9591–5. https://doi.org/10.1128/JVI.02776-06 PMID: 17553878

25. Phadungsombat J, Lin MY-C, Srimark N, Yamanaka A, Nakayama EE, Moolasart V, et al. Emergence of genotype Cosmopolitan of dengue virus type 2 and genotype III of dengue virus type 3 in Thailand. Samy AM, editor. PLOS ONE. 2018 Nov 12; 13(11):e0207220. https://doi.org/10.1371/journal.pone.0207220 PMID: 30419004

26. Salje H, Lessler J, Maljkovic Berry I, Melendrez MC, Endy T, Kalayanarooj S, et al. Dengue diversity across spatial and temporal scales: Local structure and the effect of host population size. Science. 2017 Mar 24; 355(6331):1302–6. https://doi.org/10.1126/science.aaj9384 PMID: 28336667

27. Christenbury JG, Aw PPK, Ong SH, Schreiber MJ, Chow A, Gubler DJ, et al. A method for full genome sequencing of all four serotypes of the dengue virus. J Virol Methods. 2010 Oct; 169(1):202–6. https://doi.org/10.1016/j.jviromet.2010.06.013 PMID: 20600330

28. Gruenberg A, Woo WS, Biedrzycka A, Wright PJ. Partial Nucleotide Sequence and Deduced Amino Acid Sequence of the Structural Proteins of Dengue Virus Type 2, New Guinea C and PUO-218 Strains. J Gen Virol. 1988 Jun 1; 69(6):1391–8.

29. Lewis JA, Chang G-J, Lanciotti RS, Kinney RM, Mayer LW, Trent DW. Phylogenetic Relationships of Dengue-2 Viruses. Virology. 1993 Nov; 197(1):216–24. https://doi.org/10.1006/viro.1993.1582 PMID: 8212556

30. Anzai S, Fukuda M, Otsuka Y, Eshita Y. Nucleotide Sequence and Phylogenetic Analyses of Dengue Type 2 Virus Isolated in the Dominican Republic. Virus Genes. 2004 Oct; 29(2):219–27. https://doi.org/10.1023/B:VIRU.0000036382.77987.84 PMID: 15284482

31. Añez G, Morales-Betoulle ME, Rios M. Circulation of Different Lineages of Dengue Virus Type 2 in Central America, Their Evolutionary Time-Scale and Selection Pressure Analysis. Coffey LL, editor. PLoS ONE. 2011 Nov 4; 6(11):e27459. https://doi.org/10.1371/journal.pone.0027459 PMID: 22076162

32. Williams M, Mayer SV, Johnson WL, Chen R, Volkova E, Vilcarromero S, et al. Lineage II of Southeast Asian/American DENV-2 is associated with a severe dengue outbreak in the Peruvian Amazon. Am J Trop Med Hyg. 2014 Sep; 91(3):611–20. https://doi.org/10.4269/ajtmh.13-0600 PMID: 25002298

33. Wu W, Bai Z, Zhou H, Tu Z, Fang M, Tang B, et al. Molecular epidemiology of dengue viruses in southern China from 1978 to 2006. Virol J. 2011 Jun 26; 8:322.

34. Zhao H, Zhang F-C, Zhu Q, Wang J, Hong W-X, Zhao L-Z, et al. Epidemiological and Virological Characterizations of the 2014 Dengue Outbreak in Guangzhou, China. PloS One. 2016; 11(6):e0156548. https://doi.org/10.1371/journal.pone.0156548 PMID: 27257804

35. Ma X, Zhen W, Yang P, Sun X, Nie W, Zhang L, et al. First confirmation of imported dengue virus serotype 2 complete genome in urine from a Chinese traveler returning from India. Virol J. 2014 Mar 25; 11:56. https://doi.org/10.1186/1743-422X-11-56 PMID: 24666930

36. Dash PK, Sharma S, Soni M, Agarwal A, Parida M, Rao PVL. Complete genome sequencing and evolutionary analysis of Indian isolates of Dengue virus type 2. Biochem Biophys Res Commun. 2013 Jul 5; 436(3):478–85. https://doi.org/10.1016/j.bbrc.2013.05.130 PMID: 23756811

37. Ng L-C, Chem Y-K, Koo C, Mudin RNB, Amin FM, Lee K-S, et al. 2013 dengue outbreaks in Singapore and Malaysia caused by different viral strains. Am J Trop Med Hyg. 2015 Jun; 92(6):1150–5. https://doi.org/10.4269/ajtmh.14-0588 PMID: 25846296

38. Moore PR, van den Hurk AF, Mackenzie JS, Pyke AT. Dengue viruses in Papua New Guinea: evidence of endemicity and phylogenetic variation, including the evolution of new genetic lineages. Emerg Microbes Infect. 2017 Dec 20; 6(12):e114. https://doi.org/10.1038/emi.2017.103 PMID: 29259329

39. Akram M, Fatima Z, Purdy MA, Sue A, Saleem S, Amin I, et al. Introduction and evolution of dengue virus type 2 in Pakistan: a phylogeographic analysis. Virol J. 2015 Sep 22; 12:148. https://doi.org/10.1186/s12985-015-0371-8 PMID: 26395339

40. Grant D, Tan GK, Qing M, Ng JKW, Yip A, Zou G, et al. A single amino acid in nonstructural protein NS4B confers virulence to dengue virus in AG129 mice through enhancement of viral RNA synthesis. J Virol. 2011 Aug; 85(15):7775–87. https://doi.org/10.1128/JVI.00665-11 PMID: 21632767

41. Hapuarachchi HC, Koo C, Rajarethinam J, Chong C-S, Lin C, Yap G, et al. Epidemic resurgence of dengue fever in Singapore in 2013–2014: A virological and entomological perspective. BMC Infect Dis. 2016 Dec; 16(1).

42. Chen H-L, Lin S-R, Liu H-F, King C-C, Hsieh S-C, Wang W-K. Evolution of dengue virus type 2 during two consecutive outbreaks with an increase in severity in southern Taiwan in 2001–2002. Am J Trop Med Hyg. 2008 Oct; 79(4):495–505. PMID: 18840735

43. Hamel R, Surasombatpattana P, Wichit S, Dauvé A, Donato C, Pompon J, et al. Phylogenetic analysis revealed the co-circulation of four dengue virus serotypes in Southern Thailand. Roques P, editor. PLOS ONE. 2019 Aug 15; 14(8):e0221179. https://doi.org/10.1371/journal.pone.0221179 PMID: 31415663

44. Huang J-H, Su C-L, Yang C-F, Liao T-L, Hsu T-C, Chang S-F, et al. Molecular characterization and phylogenetic analysis of dengue viruses imported into Taiwan during 2008–2010. Am J Trop Med Hyg. 2012 Aug; 87(2):349–58. https://doi.org/10.4269/ajtmh.2012.11-0666 PMID: 22855770

45. Afreen N, Naqvi IH, Broor S, Ahmed A, Kazim SN, Dohare R, et al. Evolutionary Analysis of Dengue Serotype 2 Viruses Using Phylogenetic and Bayesian Methods from New Delhi, India. Scarpino SV, editor. PLoS Negl Trop Dis. 2016 Mar 15; 10(3):e0004511. https://doi.org/10.1371/journal.pntd.0004511 PMID: 26977703

46. Lee K-S, Lai Y-L, Lo S, Barkham T, Aw P, Ooi P-L, et al. Dengue Virus Surveillance for Early Warning, Singapore. Emerg Infect Dis. 2010 May; 16(5):847–9. https://doi.org/10.3201/eid1605.091006 PMID: 20409381

47. Ali A, Ali I. The Complete Genome Phylogeny of Geographically Distinct Dengue Virus Serotype 2 Isolates (1944–2013) Supports Further Groupings within the Cosmopolitan Genotype. Alvisi G, editor. PLOS ONE. 2015 Sep 28; 10(9):e0138900. https://doi.org/10.1371/journal.pone.0138900 PMID: 26414178

48. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016; 33(7):1870–4. https://doi.org/10.1093/molbev/msw054 PMID: 27004904

49. Messina JP, Brady OJ, Scott TW, Zou C, Pigott DM, Duda KA, et al. Global spread of dengue virus types: mapping the 70 year history. Trends Microbiol. 2014; 22(3):138–46. https://doi.org/10.1016/j.tim.2013.12.011 PMID: 24468533

50. Ty Hang TT, Holmes EC, Duong V, Nguyen TQ, Tran TH, Quail M, et al. 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. 2010; 4.

51. Lambrechts L. Quantitative genetics of Aedes aegypti vector competence for dengue viruses: towards a new paradigm? Trends Parasitol. 2011; 27(3):111–4. https://doi.org/10.1016/j.pt.2010.12.001 PMID: 21215699

52. Lambrechts L, Chevillon C, Albright RG, Thaisomboonsuk B, Richardson JH, Jarman RG, et al. Genetic specificity and potential for local adaptation between dengue viruses and mosquito vectors. BMC Evol Biol. 2009; 9(160):1471–2148.

53. Weaver SC, Vasilakis N. Molecular evolution of dengue viruses: contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. Infect Genet Evol. 2009; 9(4):523–40. https://doi.org/10.1016/j.meegid.2009.02.003 PMID: 19460319

54. Messer WB, Gubler DJ, Harris E, Sivananthan K, Silva AM. Emergence and global spread of a dengue serotype 3, subtype III virus. Emerg Infect Dis. 2003;9.

55. Shrivastava S, Tiraki D, Diwan A, Lalwani SK, Modak M, Mishra AC, et al. Co-circulation of all the four dengue virus serotypes and detection of a novel clade of DENV-4 (genotype I) virus in Pune, India during 2016 season. Ansari AA, editor. PLOS ONE. 2018 Feb 22; 13(2):e0192672. https://doi.org/10.1371/journal.pone.0192672 PMID: 29470509

**56.** Dash PK, Parida MM, Saxena P, Abhyankar A, Singh CP, Tewari KN, et al. Reemergence of dengue virus type-3 (subtype-III) in India: implications for increased incidence of DHF & DSS. Virol J. 2006 Jul 6; 3:55. https://doi.org/10.1186/1743-422X-3-55 PMID: 16824209

**57.** Rico-Hesse R, Harrison LM, Salas RA, Tovar D, Nisalak A, Ramos C, et al. Origins of Dengue Type 2 Viruses Associated with Increased Pathogenicity in the Americas. Virology. 1997 Apr; 230(2):244–51. https://doi.org/10.1006/viro.1997.8504 PMID: 9143280

**58.** Rico-Hesse R. Microevolution and virulence of dengue viruses. Adv Exp Med Biol. 2003; 59:315–41.