

Title Page

• **Natural infection of cynomolgus monkeys with dengue virus occurs in epidemic cycles in the Philippines**

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• **Short running title: Natural infection of cynomolgus monkeys with DENV**

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Summary

To investigate the potential role of non-human primates (NHPs) in a dengue virus (DENV) epidemic, we conducted serological and genomic studies using plasma samples collected from 100 cynomolgus monkeys (*Macaca fascicularis*) in an animal breeding facility in the Philippines. An ELISA revealed 21 monkeys with a positive IgM reaction, and 19 positive for IgG. Five of the monkeys were positive for both IgM and IgG. Of the 21 IgM-positive samples, a neutralisation assay identified seven containing DENV-specific antibodies. We amplified the viral non-structural 1 (NS1) gene (2/7) and the envelope (E) gene (1/7) of these seven samples, by RT-PCR. Phylogenetic analyses revealed that these DENV genes belonged to the epidemic DENV-2 family, not the sylvatic DENV family. The results suggest that NHPs may serve as a reservoir of epidemic DENV; therefore, the ecology of the urban DENV infection cycle should be investigated in detail.

Text

Dengue virus (DENV), a flavivirus consisting of four serotypes (DENV-1 through to DENV-4), is the causative agent of dengue fever and dengue haemorrhagic fever (DF and DHF, respectively), which have a great impact on public health in the tropics and subtropics. DENV, an arthropod-borne virus, whose infection cycle is maintained between human and *Aedes aegypti* mosquitos, prevails in urban areas containing substantial human populations (urban DENV or epidemic DENV) (Gubler, 1998). However, DENV infection in wild non-human primates (NHPs) has also been demonstrated, and can be detected by amplifying the viral genome, detecting the antibody or recovering the infectious virus from blood (Inoue *et al.*, 2003; Vasilakis *et al.*, 2007; Vasilakis *et al.*, 2008; Vasilakis *et al.*, 2011; Rossi *et al.*, 2012). Phylogenetically, DENV in NHPs is distant from that causing urban DENV, suggesting the existence of an infection cycle between NHPs and canopy-dwelling mosquitos, which allows maintenance of such a virus (sylvatic DENV). It is proposed that urban DENV evolved from sylvatic DENV by host range expansion to *Aedes* mosquitos. Some evidence suggests that occasional transmission of sylvatic DENV to humans occurs, mainly due to encounters by people living in the vicinity of the forest with mosquitos carrying sylvatic DENV (Vasilakis *et al.*, 2007; Vasilakis *et al.*, 2008; Vasilakis *et al.*, 2008; Cardoso *et al.*, 2009; Durbin *et al.*, 2013). Because it can be transmitted to humans, sylvatic DENV poses a potential epidemic risk. Conversely, one report suggests that some “spill back” of urban DENV to NHPs occurs, implying that these animals could serve as a reservoir of urban DENV (Teoh *et al.*, 2010).

To understand the role of NHPs in a DENV epidemic, we conducted serological and genomic

60 studies of plasma samples collected from cynomolgus monkeys (*Macaca fascicularis*), housed in an
61 animal breeding facility in the Philippines, where DENV is endemic.

62 The plasma samples were collected from 100 cynomolgus monkeys in April, 2010. Of the 100
63 animals included in the current study, 20 were randomly selected from a group of animals that had
64 recently entered the facility from the wild, designated new-intake breeders (NIBs); 60 were selected
65 from another group of animals that had been in the facility for longer than the NIBs, designated feral
66 breeders (FBs); and the other 20 were selected from an additional group consisting of animals that were
67 born and raised in the facility, designated F1/F2 breeders (F1/F2Bs). Because assaying anti-DENV
68 immunoglobulin is an established method of determining the infectious status of a patient, it was
69 applied to the monkey samples. We performed an enzyme-linked immunosorbent assay (ELISA;
70 Dengue Virus IgG DxSelect, Dengue Virus IgM Capture DxSelect, FOCUS) to detect IgM, which is
71 induced earlier, and IgG, which is induced later during infection, in the plasma. Samples with
72 absorbance greater than twice that of the negative control (P/N ratio >2.00) were considered positive.
73 Plasma samples from 21 monkeys were IgM-positive, and 19 were IgG-positive. Five samples were
74 positive for both IgM and IgG (Table1). There was no significant difference in the seroprevalence of
75 DENV by IgM or IgG among the NIB, FB and F1/F2B animal groups (data not shown).

76 Individuals infected with a flavivirus produce antibodies that are broadly reactive to other
77 flaviviruses, in addition to serotype-specific neutralising antibodies (Gubler, 1998). ELISA, in principle,
78 does not specifically detect such neutralising antibodies. Therefore, the presence of antibodies

79 identified by ELISA does not necessarily prove DENV infection in monkeys. It has been established
80 that the plaque reduction neutralisation test (PRNT) is highly specific for differentiation of serotypes of
81 particular flavivirus infections, including DENV. Therefore, to identify genuine DENV infection in the
82 positive samples, we conducted titration of monkey plasma samples with the PRNT using BHK-21 cells
83 with four DENV isolates representing individual serotypes: for DENV-1, strain 02-20, which was
84 isolated in 2002 from a Japanese traveller manifesting DF with a history of travel to Thailand (Tajima *et*
85 *al.*, 2006; Accession No. AB178040); for DENV-2, strain 16681, which was isolated in 1964 from a
86 patient with DHF in Thailand (Kinney *et al.*, 1997; Accession No. U87411); for DENV-3, strain 00-40,
87 which was isolated in 2000 from a Japanese traveller returning from Thailand (Ito *et al.*, 2007;
88 Accession No. AB111082); for DENV-4, strain 09-48, which was isolated in 2009 from a Japanese
89 traveller returning from Indonesia (unpublished and provided as a gift from the National Institute of
90 Infectious Diseases, Japan (NIID)), following the protocol developed by NIID. Plasma collected from
91 DENV-negative rhesus monkeys served as a negative control and diluent for the test plasma samples.
92 Of 35 ELISA-positive samples, the PRNT identified nine that contained DENV-specific antibodies;
93 three containing antibodies against DENV-1, five containing antibodies against DENV-2, and one
94 containing antibodies against DENV-3. Using the diagnostic criterion of neutralisation at more than
95 1:10 of the reciprocal dilution of plasma samples, none of the samples contained antibodies directed
96 against multiple DENV serotypes. The remaining 26 ELISA-positive samples neutralised none of the
97 DENV serotypes.

98 It is generally accepted that IgM is generated 6-14 days post-DENV infection, followed by
99 IgG. The presence of IgM alone in plasma indicates acute infection. Plasma containing both IgM and
100 IgG indicates a sub-acute infection, and plasma containing only IgG indicates a past infection. Plasma
101 #9173, which yielded absorbance values of 2.74 for IgM and 4.94 for IgG in the ELISA, and a
102 neutralisation titre of 1:465 for DENV-1 in the PRNT, was diagnosed as an acute/sub-acute DENV-1
103 infection with a history of other flavivirus infections. Plasma #4409-A, which yielded absorbance
104 values of 3.80 for IgM and 3.59 for IgG in the ELISA, and neutralisation titre of 1:25 for DENV-2 in the
105 PRNT, was diagnosed as a DENV-2 infection with a history of other flavivirus infections or sub-acute
106 DENV-2 infections. The plasma samples 4477, 8136, 7651 and 2716-C, which yielded high absorbance
107 for IgM and comparable or lower absorbance for IgG in the ELISA, and in which we detected
108 DENV-specific neutralising antibodies by the PRNT, were diagnosed as acute DENV infections. The
109 plasma samples 12860 and 6274, which yielded high absorbance for IgG but not IgM, and in which we
110 detected DENV-specific neutralising antibodies by the PRNT, were diagnosed as having had previous
111 infections with DENV-1 or DENV-2, respectively.

112 We were unable to recover infectious virus from plasma samples containing high IgM and
113 DENV-specific neutralising antibody titres that were likely to be from an acute DENV infection with
114 viraemia. As an alternative, we attempted to amplify the viral genes from these samples by PCR to
115 characterise the viruses. Complementary DNA was synthesised from total RNA extracted from the
116 plasma samples by reverse transcription, and amplified by PCR using primers described previously

(Yenchitsomanus *et al.*, 1996). The PCR product was sequenced directly without a cloning step.

We successfully amplified part of the non-structural 1 (NS1) gene (approximately 200 bp) from samples #8136 and #8361 (GenBank accession numbers, AB828676 and AB828677, respectively), and part of the envelope (E) gene, spanning domains 2 and 3 (approximately 300 bp), from sample #8361 (GenBank accession number, AB828678). Nucleotide sequence alignments of the sequences amplified in the current study with those published previously are provided in the Supplementary Materials. We utilised these sequences to construct phylogenetic trees using the maximum-likelihood method (Felsenstein, 1981). Phylogenetic analysis of the NS1 gene revealed that the viruses in samples #8136 and #8361 belonged to DENV-2, in the cluster of urban DENV strains isolated from patients in Asian countries (Figure 1). The homology between nucleotide sequences #8136 and #8361 was 96.5%, indicating that they are independent strains. Interestingly, these sequences amplified from the monkeys were distinct from the sylvatic DENV strains reported previously. Because no sequences of the NS1 gene from strains recently isolated in the Philippines were available, we next analysed the E gene, which has been studied extensively, and multiple sequences from Filipino isolates were available for comparison.

We successfully amplified the E gene from only the specimen #8361. Analysis of the amplicon also showed it to be from an urban DENV strain, not a sylvatic one, which was related to strains that circulated in Asia in 2010 (Warrilow *et al.*, 2012). The viral sequence from animal #8361 formed a cluster with strain 16681, which was used as a reference strain in our laboratory, but was

distant from Filipino strains that formed a unique cluster with isolates recovered in Asian countries in 2010, although the nucleotide diversity between the two clusters was 1.9% (Figure 2).

A higher homology of the E gene sequence from specimen #8361 to the reported sequence of strain 16681 could result from carryover of the 16681 viral material into the amplification step of PCR, although these sequences bore unique nucleotides (Supplementary Materials). Therefore, to ascertain that the amplified sequences were indeed from the plasma samples, and not contamination by our laboratory strain, we analysed the sequences of corresponding regions in 16681 by single genome amplification (Salazar-Gonzalez *et al.*, 2008). We synthesised complementary DNA from total RNA extracted from the 16681 virus stock by reverse transcription, and amplified the genomic region by PCR using a diluted DNA template. We obtained five identical SGA clones that differed from those amplified from the plasma samples. The nucleotide sequence of the SGA clones was not identical to that deposited in the database, although the phylogenetic relationships among them were the closest among the sequences analysed (data not shown). Therefore, we concluded that these sequences were indeed amplified from the monkey plasma samples. Based on the above results, we concluded that the cynomolgus monkeys were infected with epidemic DENV.

In the Philippines, a remarkably high number of DENV cases was reported in 2010, more than triple the number reported in 2009. The DENV epidemic had already started by April 2010, when we collected the blood samples from the animals. The Department of Health of the Republic of the Philippines issued an early warning regarding the DENV epidemic, because the number of cases

exceeded the Alert and Epidemic thresholds (Republic of the Philippines Department of health, Disease Surveillance Report: Dengue cases, morbidity week 43, 2010). Our seroprevalence result (seven positive DENV samples out of 100), which was somewhat higher than that reported previously (Inoue *et al.*, 2003), might have been influenced by the extensive epidemic in the country at that time.

We suggest that animals #9173 and #4409-A, which harboured relatively high IgM and IgG titres, but DENV-specific neutralising antibody directed against only one serotype, had been infected with another flavivirus, prior to exposure to DENV-1 and -2, respectively. Among the 21 samples positive for IgM, the PRNT found seven positive and 14 negative for DENV. These 14 animals may have been infected by another flavivirus. A previous study in the Philippines found that the prevalence of Japanese encephalitis virus (JEV) in cynomolgus monkeys was 35.2% as detected by IgM ELISA (Inoue *et al.*, 2003), and so our samples might also have been infected with JEV. Our understanding of the nature of flavivirus infection would be enhanced by surveying other flaviviruses pathogenic to humans, in addition to DENV, among NHPs.

The FB animals #8136 and #8361, estimated to be 12 and 11 years old, respectively, may have been exposed to DENV in the facility, as they had been in residence for 7 years and 3 months and 6 years and 4 months, respectively. The physical proximity of the animal facility, which is located 800 metres from the nearest human residence, 3.5 kilometres from a community, and in the vicinity of the Metro Manila area where urban DENV is epidemic, might have provided the conditions for the observed “spillback” of epidemic DENV to the NHPs. Our results suggest that NHPs could serve as a

174 reservoir of epidemic DENV, and we should therefore be vigilant and increase our understanding of the

175 ecology of the urban DENV infection cycle.

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251

252 Table 1. Results of the serological assays.

Monkey ID	Category	ELISA (O.D. _{450nm})		Reciprocal neutralisation titres by PRNT ₅₀ *			
		IgM	IgG	DENV-1	DENV-2	DENV-3	DENV-4
12860	NIBs†	1.36	8.21	1:127	<1:10	<1:10	<1:10
12899	NIBs	1.49	7.15	<1:10	<1:10	<1:10	<1:10
12862	NIBs	3.33	0.99	<1:10	<1:10	<1:10	<1:10
12873	NIBs	2.70	1.30	<1:10	<1:10	<1:10	<1:10
9672	FBs‡	2.21	1.05	<1:10	<1:10	<1:10	<1:10
6557	FBs	2.32	0.96	<1:10	<1:10	<1:10	<1:10
6554	FBs	1.10	3.73	<1:10	<1:10	<1:10	<1:10
6274	FBs	1.80	4.33	<1:10	1:28	<1:10	<1:10
9339	FBs	2.41	2.02	<1:10	<1:10	<1:10	<1:10
9340	FBs	1.88	2.54	<1:10	<1:10	<1:10	<1:10
5945	FBs	2.55	1.31	<1:10	<1:10	<1:10	<1:10
4477	FBs	3.81	1.06	<1:10	<1:10	1:18	<1:10
8136	FBs	2.13	2.35	<1:10	1:76	<1:10	<1:10
9173	FBs	2.74	4.94	1:465	<1:10	<1:10	<1:10
9171	FBs	2.56	1.05	<1:10	<1:10	<1:10	<1:10
8974	FBs	5.02	0.96	<1:10	<1:10	<1:10	<1:10
8386	FBs	3.96	0.89	<1:10	<1:10	<1:10	<1:10
6549	FBs	2.76	0.84	<1:10	<1:10	<1:10	<1:10
7698	FBs	1.25	2.70	<1:10	<1:10	<1:10	<1:10
7687	FBs	1.94	2.61	<1:10	<1:10	<1:10	<1:10
8399	FBs	1.92	3.98	<1:10	<1:10	<1:10	<1:10
7651	FBs	2.57	0.94	1:20	<1:10	<1:10	<1:10
9208	FBs	1.22	4.20	<1:10	<1:10	<1:10	<1:10
9220	FBs	2.42	1.13	<1:10	<1:10	<1:10	<1:10
6255	FBs	8.39	1.22	<1:10	<1:10	<1:10	<1:10
8361	FBs	2.59	0.89	<1:10	1:22	<1:10	<1:10
8966	FBs	1.32	3.42	<1:10	<1:10	<1:10	<1:10
8367	FBs	2.04	3.06	<1:10	<1:10	<1:10	<1:10
1095-J	F1/F2Bs§	2.86	0.90	<1:10	<1:10	<1:10	<1:10
1558-C	F1/F2Bs	1.27	2.57	<1:10	<1:10	<1:10	<1:10
S-6941-C	F1/F2Bs	1.81	2.44	<1:10	<1:10	<1:10	<1:10
2354-B	F1/F2Bs	1.68	2.66	<1:10	<1:10	<1:10	<1:10
4409-A	F1/F2Bs	3.80	3.59	<1:10	1:25	<1:10	<1:10
2716-C	F1/F2Bs	2.80	1.26	<1:10	1:17	<1:10	<1:10

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254 Monkeys with IgM and/or IgG reactive to DENV antigen as determined by ELISA are listed. Samples

255 positive for DENV-specific neutralising antibody by plaque reduction neutralisation test (PRNT) are

256 indicated in bold. *PRNT₅₀, 50% plaque reduction neutralisation titre; † NIBs, new-intake breeders;

257 ‡FBs, feral breeders; §F1/F2Bs, F1/F2 breeders.

258

Figure legends

Fig. 1. Phylogenetic relationship of DENV from cynomolgus monkeys to other isolates based on the sequence of the non-structural 1 (NS1) gene.

Viral sequences identified in this study (#8136 and 8361) are enclosed in rectangles. The phylogenetic tree was constructed by the maximum-likelihood method (Felsenstein, 1981).

Fig. 2. Phylogenetic relationship of DENV from a cynomolgus monkey to other isolates based on the sequence of the envelope (E) gene.

The viral sequence identified in this study (#8361) is enclosed in a rectangle. The phylogenetic tree was constructed by the maximum-likelihood method (Felsenstein, 1981).

Captions for Supplementary Materials 1

Supplementary Figure 1.

Nucleotide alignment of a portion of the NS1 region amplified from the plasma of cynomolgus monkeys (#8361 and #8136, boxed) and those from reference DENV strains. Nucleotides substituted from Malaysia.M2/2004 are depicted as symbols (A, T, G, or C). Identical nucleotides are depicted as dashes (-).

Supplementary Figure 2.

Nucleotide alignment of a portion of the E region amplified from the plasma of a cynomolgus monkey (#8361, boxed) and those from reference DENV strains. Nucleotides substituted from JN568264 Philippines 2010 are depicted as symbols (A, T, G, or C). Identical nucleotides are depicted as dashes (-).

287

Supplementary material 1

288

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1                                     100
Malaysia.M2/2004 AAAATCACACACCTCTGGAGCAATGGAGTGTAGAAAGTGAGATGATAATCCAAAGAATTTCGCTGGACCAAGTGTACACACAACTATAGACCAGGC
#8361 -----T-----C-----T-----C-----
#8136 T--G---TTTAT--A-----C-----T-----C-----
16681 -----C-----T-----C-----
TH_0038_74 -----C-----T-----C-----
US/BID-V4825/2010 ---GC-----T---A-----C-----
Indonesia.TB161/2004 ---G-----T-----T-----T---A-----T---C-----
Austraria.TSV01/1993 ---G-----T-----C-----T---A-----T---C-----
TH/BID-V2156/2001 ---G-----T-----C-----T-----T-----C-----
Malaysia.DKD811 Sylvertic strain --G-----C---G---A-----A-----C---G---C-----A-----T---C---C---
Malaysia.P8-1407 Sylvertic Strain --G-----T-----T-----C---G---A-----A-----CC-G-C-----A---G---T---C---G---T---

101                                     200
Malaysia.M2/2004 TACCATACACAAATAGCAGGACCATGGCATTAGGTAAGCTTGAGATGGACTTTGATTCTCGCATGGAACACAGTGTTGTGACTGAGGACTGCGGAA
#8361 -----C-----A-----A-----G-----
#8136 -----C-----T-----A-----A-----G-----
16681 -----A-----C-----T-----A-----A-----
TH_0038_74 -----C-----C-----A-----A-----T-----
US/BID-V4825/2010 -----C-----C-----A-----C-----T-----
Indonesia.TB161/2004 --T-----CG-----C-----C---G-----A-----C-----G-----T-----
Austraria.TSV01/1993 --T-----CG-----C-----G-----A-----C-----A-----T-----
TH/BID-V2156/2001 -----C-----C-----A-----C-----A-----A-----
Malaysia.DKD811 Sylvertic strain --T-----C---C-----C---G---C---A---T---T---AC-G-T-G---C---G---A---G---A---T---
Malaysia.P8-1407 Sylvertic Strain --T-C-----CC-C-----C---G---C-GA---T---T---C---AC-T---G---T---C---G---A---G---C---A---T---

201                                     214
Malaysia.M2/2004 ATAGAGGACCCCTGT
#8361 -----C-
#8136 -----C-
16681 -----C-
TH_0038_74 -----C-
US/BID-V4825/2010 -----C-
Indonesia.TB161/2004 -----C-
Austraria.TSV01/1993 -C-----C-
TH/BID-V2156/2001 -----C-
Malaysia.DKD811 Sylvertic strain -----A-CC
Malaysia.P8-1407 Sylvertic Strain -----A-CC
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Supplementary material 2

	1	100
JN568264 Philippines 2010	GCACTCACAGGGGCCACGGAATCCAGATGTCATCAGGAACTTACTGTTACAGGACATCTTAAGTCAGGCTGAGAATGGACAACTACAGCTCAAAG	
#8361	-----A-----A-----T-----C-----G-----T-----	
16681	-----T-----A-----A-----C-----C-----C-----G-----	
SGA clone 16681	-----A-----AG-----C-----C-----G-----G-----T-----	
JN568276 Southeast Asia 2010	-----A-----A-----A-----G-----C-----T-----C-----G-----T-----	
JN568263 Philippines 2003	-----A-----A-----A-----G-----C-----T-----C-----G-----T-----	
01-04-IHuNIID Indonesia 2001-2002	-----A-----A-----A-----G-----C-----T-----C-----G-----T-----	
JN 568246 Australia 2010	-----A-----A-----A-----G-----C-----T-----C-----G-----T-----	
DKD811/2008 Malaysia 2008 sylvatic strain	-----T-----A-----A-----A-----T-----A-----T-----T-----G-----G-----A-----	
P8-1407 Malaysia 1970 sylvatic strain	-----T-----A-----A-----A-----T-----A-----T-----T-----G-----G-----A-----	
	101	200
JN568264 Philippines 2010	GAATGTCATATTTCTATGTGTACAGGAAAGTTTAAAGTTGTGAAGGAAATAGCAGAAACACAAATGGAACAATAGTAATCAGAGTGCAATATGAAGGGGA	
#8361	-----C-----C-----G-----T-----	
16681	-----C-----C-----T-----	
SGA clone 16681	-----C-----C-----T-----	
JN568276 Southeast Asia 2010	-----C-----C-----G-----T-----	
JN568263 Philippines 2003	-----C-----C-----T-----A-----	
01-04-IHuNIID Indonesia 2001-2002	-----C-----C-----T-----A-----	
JN 568246 Australia 2010	-----C-----C-----G-----T-----	
DKD811/2008 Malaysia 2008 sylvatic strain	-----C-----C-----C-----C-----A-----T-----G-----G-----C-----T-----G-----A-----	
P8-1407 Malaysia 1970 sylvatic strain	-----C-----C-----G-----C-----C-----A-----T-----G-----G-----C-----T-----G-----A-----	
	201	250
JN568264 Philippines 2010	CGGTTCTCCGTGCAAGATCCCTTTTGAAATAATGGATTTGGAAAAAGAC	
#8361	-----C-----A-----T-----A-----G-----	
16681	-----C-----A-----G-----	
SGA clone 16681	-----C-----A-----T-----G-----	
JN568276 Southeast Asia 2010	-----C-----A-----A-----G-----T-----	
JN568263 Philippines 2003	-----C-----A-----A-----G-----T-----	
01-04-IHuNIID Indonesia 2001-2002	-----C-----A-----A-----G-----T-----	
JN 568246 Australia 2010	-----C-----A-----T-----A-----G-----T-----	
DKD811/2008 Malaysia 2008 sylvatic strain	A--AG-A--A-----T-----C-----G-----C-----G--AG-	
P8-1407 Malaysia 1970 sylvatic strain	A--G-A--A-----T-----C-----G-----C-----G--AG-	

293

294

295

296

1
2

3
4

Figure 1

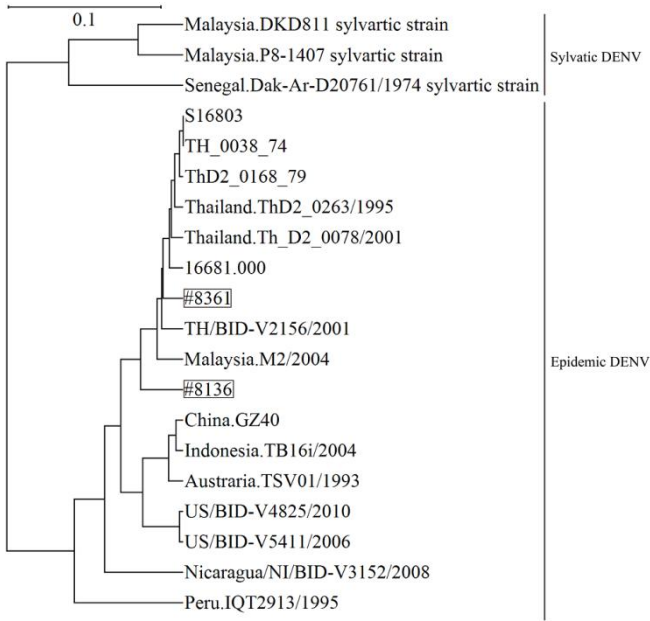


Figure 2

