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Short Communication

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

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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 neutralization assay identified seven containing DENV-specific antibodies. We amplified the viral non-structural 1 (NS1) gene in two and the envelope (E) gene in one 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. These 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 these animals in detail.

Dengue virus (DENV), a flavivirus consisting of four serotypes (DENV-1–4), is the causative agent of dengue fever and dengue haemorrhagic fever, 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 infectious virus from blood (Inoue *et al.*, 2003; Vasilakis *et al.*, 2007a, 2008a, 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.*, 2007b, 2008b; Cardosa *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 'spillback' 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 studies of plasma samples collected from cynomolgus monkeys (*Macaca fascicularis*), housed in an animal breeding facility in the Philippines where DENV is endemic.

The plasma samples were collected from 100 cynomolgus monkeys in April 2010. Of the 100 animals included in the current study, 20 were selected randomly from a group of animals that had recently entered the facility from the wild, designated new-intake breeders (NIBs); 60 were selected from another group of animals that had been in the facility for longer than the NIBs, designated feral breeders (FBs); and the other 20 were selected from an additional group consisting of animals that were born and raised in the facility, designated F1/F2 breeders (F1/F2Bs). Because assaying anti-DENV immunoglobulin is an established method of determining the infectious status of a patient, it was applied to the monkey samples. We performed an ELISA (Dengue Virus IgG DxSelect, Dengue Virus IgM Capture DxSelect, Focus Doagnostics) to detect IgM, which is induced earlier, and IgG, which is induced later during infection, in the plasma. Samples with absorbance greater than twice that of the negative control (positive : negative ratio >2.00) were considered positive. Plasma samples

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are AB828676–AB828678.

Two supplementary figures are available with the online version of this paper.

from 21 monkeys were IgM positive and from 19 were IgG positive. Five samples were positive for both IgM and IgG (Table 1). There was no significant difference in the seroprevalence of DENV by IgM or IgG among the NIB, FB and F1/F2B animal groups (data not shown).

Individuals infected with a flavivirus produce antibodies that are broadly reactive to other flaviviruses, in addition to serotype-specific neutralizing antibodies (Gubler, 1998). ELISA, in principle, does not specifically detect such neutralizing antibodies. Therefore, the presence of antibodies identified by ELISA does not necessarily prove DENV infection in monkeys. It has been established that a plaque reduction neutralization test (PRNT) is highly specific for differentiation of serotypes of particular flavivirus infections, including DENV. Therefore, to identify genuine DENV infection in the positive samples, we conducted titration of monkey plasma samples with a PRNT using BHK-21 cells with four DENV isolates representing individual serotypes: for DENV-1, strain 02-20, which was isolated in 2002 from a Japanese traveller manifesting dengue fever with a history of travel to Thailand (Tajima *et al.*, 2006; GenBank accession no. AB178040); for DENV-2, strain 16681, which was isolated in 1964 from a patient with dengue haemorrhagic fever in Thailand (Kinney *et al.*, 1997; GenBank accession no. U87411); for DENV-3, strain 00-40, which was isolated in 2000 from a Japanese traveller returning from Thailand (Ito *et al.*, 2007; GenBank accession no. AB111082); and for DENV-4, strain 09-48, which was isolated in 2009 from a

Table 1. Results of the serological assays

Monkeys with IgM and/or IgG reactive to DENV antigen as determined by ELISA are listed. Samples positive for DENV-specific neutralizing antibody by a 50 % PRNT ($PRNT_{50}$) are indicated in bold.

Monkey ID	Category	ELISA (A_{450})		Reciprocal neutralization titres by $PRNT_{50}$			
		IgM	IgG	DENV-1	DENV-2	DENV-3	DENV-4
12860	NIB	1.36	**8.21**	**1 : 127**	<1 : 10	<1 : 10	<1 : 10
12899	NIB	1.49	**7.15**	<1 : 10	<1 : 10	<1 : 10	<1 : 10
12862	NIB	**3.33**	0.99	<1 : 10	<1 : 10	<1 : 10	<1 : 10
12873	NIB	**2.70**	1.30	<1 : 10	<1 : 10	<1 : 10	<1 : 10
9672	FB	**2.21**	1.05	<1 : 10	<1 : 10	<1 : 10	<1 : 10
6557	FB	**2.32**	0.96	<1 : 10	<1 : 10	<1 : 10	<1 : 10
6554	FB	1.10	**3.73**	<1 : 10	<1 : 10	<1 : 10	<1 : 10
6274	FB	1.80	**4.33**	<1 : 10	**1 : 28**	<1 : 10	<1 : 10
9339	FB	**2.41**	**2.02**	<1 : 10	<1 : 10	<1 : 10	<1 : 10
9340	FB	1.88	**2.54**	<1 : 10	<1 : 10	<1 : 10	<1 : 10
5945	FB	**2.55**	1.31	<1 : 10	<1 : 10	<1 : 10	<1 : 10
4477	FB	**3.81**	1.06	<1 : 10	<1 : 10	**1 : 18**	<1 : 10
8136	FB	**2.13**	**2.35**	<1 : 10	**1 : 76**	<1 : 10	<1 : 10
9173	FB	**2.74**	**4.94**	**1 : 465**	<1 : 10	<1 : 10	<1 : 10
9171	FB	**2.56**	1.05	<1 : 10	<1 : 10	<1 : 10	<1 : 10
8974	FB	**5.02**	0.96	<1 : 10	<1 : 10	<1 : 10	<1 : 10
8386	FB	**3.96**	0.89	<1 : 10	<1 : 10	<1 : 10	<1 : 10
6549	FB	**2.76**	0.84	<1 : 10	<1 : 10	<1 : 10	<1 : 10
7698	FB	1.25	**2.70**	<1 : 10	<1 : 10	<1 : 10	<1 : 10
7687	FB	1.94	**2.61**	<1 : 10	<1 : 10	<1 : 10	<1 : 10
8399	FB	1.92	**3.98**	<1 : 10	<1 : 10	<1 : 10	<1 : 10
7651	FB	**2.57**	0.94	**1 : 20**	<1 : 10	<1 : 10	<1 : 10
9208	FB	1.22	**4.20**	<1 : 10	<1 : 10	<1 : 10	<1 : 10
9220	FB	**2.42**	1.13	<1 : 10	<1 : 10	<1 : 10	<1 : 10
6255	FB	**8.39**	1.22	<1 : 10	<1 : 10	<1 : 10	<1 : 10
8361	FB	**2.59**	0.89	<1 : 10	**1 : 22**	<1 : 10	<1 : 10
8966	FB	1.32	**3.42**	<1 : 10	<1 : 10	<1 : 10	<1 : 10
8367	FB	**2.04**	**3.06**	<1 : 10	<1 : 10	<1 : 10	<1 : 10
1095-J	F1/F2B	**2.86**	0.90	<1 : 10	<1 : 10	<1 : 10	<1 : 10
1558-C	F1/F2B	1.27	**2.57**	<1 : 10	<1 : 10	<1 : 10	<1 : 10
S-6941-C	F1/F2B	1.81	**2.44**	<1 : 10	<1 : 10	<1 : 10	<1 : 10
2354-B	F1/F2B	1.68	**2.66**	<1 : 10	<1 : 10	<1 : 10	<1 : 10
4409-A	F1/F2B	**3.80**	**3.59**	<1 : 10	**1 : 25**	<1 : 10	<1 : 10
2716-C	F1/F2B	2.80	1.26	<1 : 10	1 : 17	<1 : 10	<1 : 10

Japanese traveller returning from Indonesia [unpublished and provided as a gift from the National Institute of Infectious Diseases, Japan (NIID)], following a protocol developed by NIID. Plasma collected from DENV-negative rhesus monkeys served as a negative control and diluent for the test plasma samples. Of 35 ELISA-positive samples, the PRNT identified nine that contained DENV-specific antibodies: three containing antibodies against DENV-1, five containing antibodies against DENV-2 and one containing antibodies against DENV-3. Using the diagnostic criterion of neutralization at more than 1 : 10 of the reciprocal dilution of plasma samples, none of the samples contained antibodies directed against multiple DENV serotypes. The remaining 26 ELISA-positive samples neutralized none of the DENV serotypes.

It is generally accepted that IgM is generated 6–14 days after DENV infection, followed by IgG. The presence of IgM alone in plasma indicates acute infection, plasma containing both IgM and IgG indicates a subacute infection, and plasma containing only IgG indicates a past infection. Plasma #9173, which yielded absorbance values of 2.74 for IgM and 4.94 for IgG in the ELISA, and a neutralization titre of 1 : 465 for DENV-1 in the PRNT, was diagnosed as an acute/subacute DENV-1 infection with a history of other flavivirus infections. Plasma #4409-A, which yielded absorbance values of 3.80 for IgM and 3.59 for IgG in the ELISA, and neutralization titre of 1 : 25 for DENV-2 in the PRNT, was diagnosed as a DENV-2 infection with a history of other flavivirus infections or subacute DENV-2 infections. Plasma samples 4477, 8136, 7651 and 2716-C, which yielded high absorbance for IgM and comparable or lower absorbance for IgG in the ELISA, and in which we detected DENV-specific neutralizing antibodies by the PRNT, were diagnosed as acute DENV infections. Plasma samples 12860 and 6274, which yielded high absorbance for IgG but not IgM, and in which we detected DENV-specific neutralizing antibodies by the PRNT, were diagnosed as indicating previous infections with DENV-1 or DENV-2, respectively.

We were unable to recover infectious virus from plasma samples containing high IgM and DENV-specific neutralizing antibody titres that were likely to be from an acute DENV infection with viraemia. As an alternative, we attempted to amplify the viral genes from these samples by PCR to characterize the viruses. Complementary DNA was synthesized from total RNA extracted from the 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 (~200 bp) from samples #8136 and #8361 (GenBank accession nos AB828676 and AB828677, respectively), and part of the envelope (E) gene, spanning domains 2 and 3 (~300 bp), from sample #8361 (GenBank accession no. AB828678). Nucleotide sequence alignments of the sequences amplified in the current study with those published previously are provided in Figs S1 and S2, available in JGV Online. We utilized 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 (Fig. 1). The nucleotide identity between 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 for which multiple sequences from Filipino isolates were available for comparison.

We successfully amplified the E gene only from 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 % (Fig. 2).

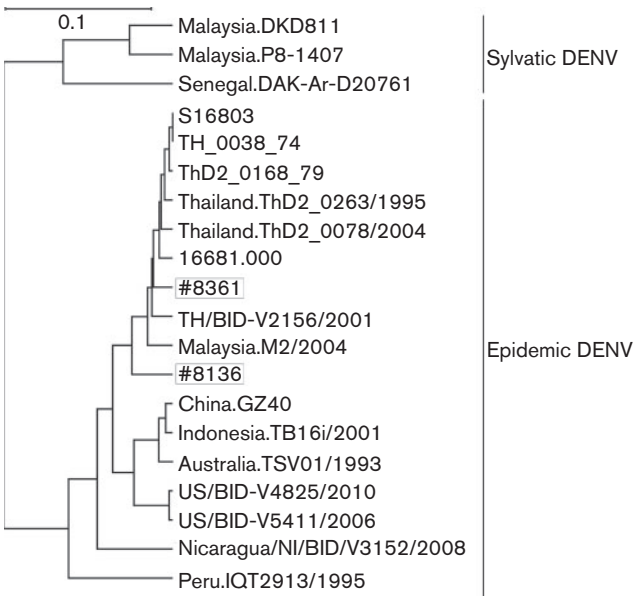

Fig. 1. Phylogenetic relationship of DENV from cynomolgus monkeys to other isolates based on the sequence of the NS1 gene. Viral sequences identified in this study (#8136 and #8361) are enclosed in rectangles. The phylogenetic tree was constructed by the maximum-likelihood method (Felsenstein, 1981). Bar, nucleotide substitutions per site.

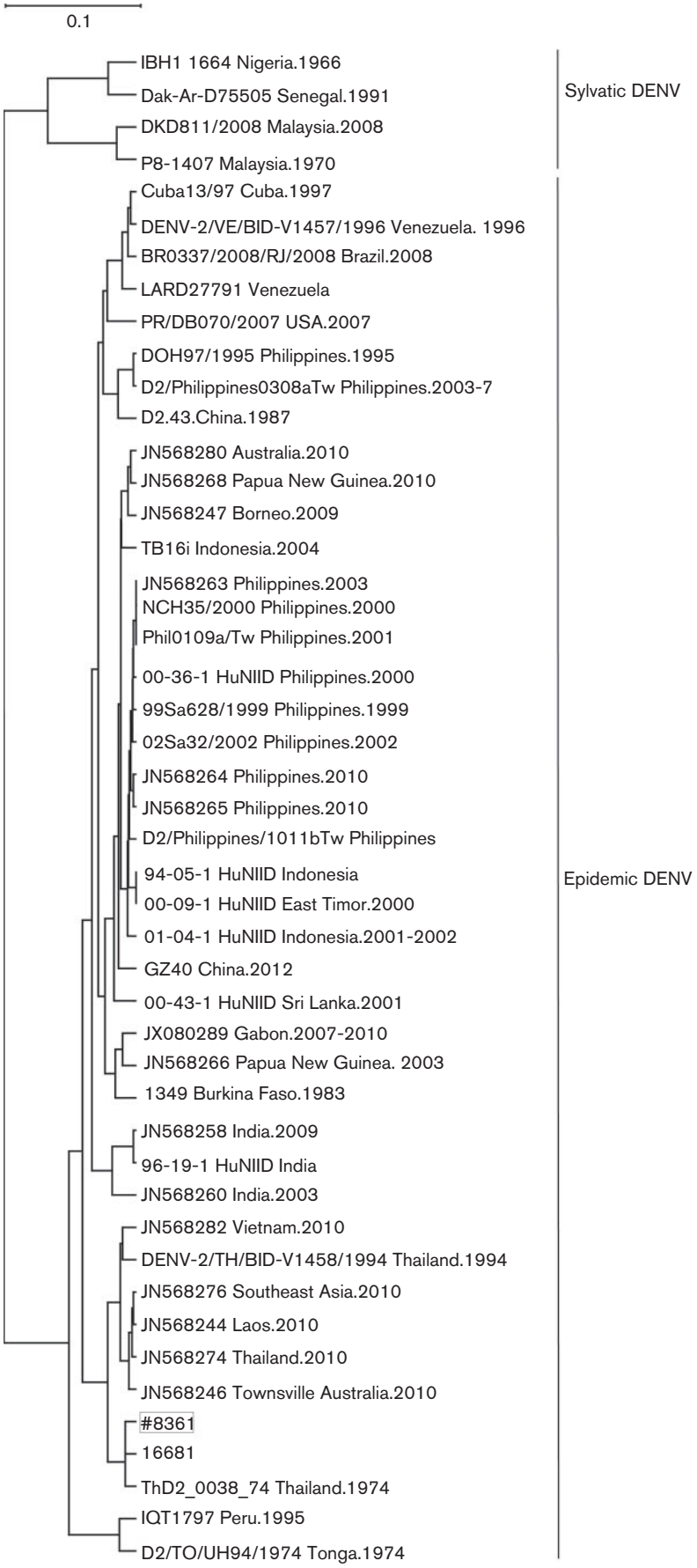

Fig. 2. Phylogenetic relationship of DENV from a cynomolgus monkey to other isolates based on the sequence of the envelope (E) gene. The viral sequence identified in this study (#8361) is enclosed in a rectangle. The phylogenetic tree was constructed by the maximum-likelihood method (Felsenstein, 1981). Bar, nucleotide substitutions per site.

A higher nucleotide identity 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 (Fig. S2). Therefore, to ascertain that the amplified sequences were indeed from the plasma samples, and not due to contamination by our laboratory strain, we analysed the sequences of corresponding regions in 16681 by single-genome amplification (SGA; Salazar-Gonzalez *et al.*, 2008). We synthesized 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 single genome amplification (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; http://www.doh.gov.ph/disease-surveillance.html). 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 neutralizing 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 in cynomolgus monkeys was 35.2 % as detected by IgM ELISA (Inoue *et al.*, 2003), so our samples may also have been infected with Japanese encephalitis virus. 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 m from the nearest human residence, 3.5 km 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 reservoir of epidemic DENV, and we should therefore be vigilant and increase our understanding of the ecology of the urban DENV infection cycle.

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