

western Mediterranean Sea. The effect of DMV infection on the health and conservation of the threatened Eurasian otter populations warrants further investigation.

### Acknowledgments

We thank Antonio Lo Polito, Maria Luigia Prencipe, and Fabio Gisoldo for their valuable technical support. We also thank Gabriella Di Francesco, Alessio Lorusso, Daniela Malatesta, and Guendalina Zaccaria for their valuable support in performing histopathological, immunohistochemical, and biomolecular analyses as well as for their greatly appreciated contribution to fruitful and insightful discussions on the data reported in the manuscript.

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## Little Evidence of Zika Virus Infection in Wild Long-Tailed Macaques, Peninsular Malaysia

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DOI: <https://doi.org/10.3201/eid2502.180258>

We tested a sample of 234 wild long-tailed macaques (*Macaca fascicularis*) trapped in Peninsular Malaysia in 2009, 2010, and 2016 for Zika virus RNA and antibodies. None were positive for RNA, and only 1.3% were seropositive for neutralizing antibodies. Long-tailed macaques are unlikely to be reservoirs for Zika virus in Malaysia.

Zika virus, first isolated from a rhesus macaque (*Macaca mulatta*) in the Zika Forest in Uganda, reemerged in the Pacific Islands and Americas in 2015 and caused unprecedented outbreaks associated with serious congenital syndromes (1). The role of animal reservoirs for Zika virus is unclear, although in Africa, nonhuman primates (NHPs) are suspected to be involved in maintaining a sylvatic cycle, as they are for 2 other flaviviruses (yellow fever and dengue viruses) also transmitted by *Aedes* mosquitoes. The presence of a sylvatic cycle for Zika virus in Africa is supported by a seroprevalence of 0%–16% in African green monkeys (*Chlorocebus sabaeus*) and vervet monkeys (*Chlorocebus*

*pygerythrus*) (2). However, even less is known about the potential role of NHPs in sylvatic cycles in Asia.

In Malaysia, Zika virus seropositivity has been reported in residents (3,4), monkeys (5), and orangutans (4), suggesting endemicity. Continual encroachment of human settlements into monkey habitats potentially increases human risk for exposure to monkey-associated zoonotic pathogens. We therefore evaluated Zika virus prevalence in long-tailed macaques (*M. fascicularis*), the most common macaque in Peninsular Malaysia, which is also widespread throughout Southeast Asia.

Staff of the Department of Wildlife and National Parks Peninsular Malaysia (also called Jabatan Perlindungan Hidupan Liar dan Taman Negara Semenanjung Malaysia [PERHILITAN]) traps monkeys foraging in human-populated areas and relocates them to deep forest areas (6). As part of PERHILITAN's Wildlife Disease Surveillance Program, serum samples were collected from 234 long-tailed macaques trapped at >30 sites throughout Malaysia in the states of Selangor, Negeri Sembilan, Perak, Pahang, Penang, and Johor (approval no. PERHILITAN JPHL&TN(IP):100-34/1.24) and stored at  $-80^{\circ}\text{C}$ . This collection comprised 145 samples acquired during October–November 2009 and October 2010 (6) and 89 acquired in March and August 2016, coinciding with the Zika virus global epidemics.

After extracting viral RNA from samples with a QIAamp Viral RNA Mini Kit (QIAGEN, <https://www.qiagen.com>), we tested samples with sufficient serum volume ( $n = 228$ ) for Zika virus envelope gene by real-time PCR (7); none were positive. We tested all 234 samples for Zika virus neutralizing antibody by 50% plaque reduction neutralization test (PRNT<sub>50</sub>) on Vero cells (Appendix, <https://wwwnc.cdc.gov/EID/article/25/2/18-0258-App1.pdf>). In total, 6 (2.6%) samples had screening Zika virus PRNT<sub>50</sub> titers  $\geq 20$  (Table); we confirmed results with a 50%

Zika virus focus reduction neutralization test (FRNT<sub>50</sub>), and samples had titers identical to or within 1 dilution of the PRNT<sub>50</sub> titer.

Because flavivirus antibodies are known to cross-react, these 6 samples were further examined for antibodies specific to the major known circulating flaviviruses in Malaysia, dengue virus serotypes 1 (DENV-1) and 2 (DENV-2), by FRNT<sub>50</sub>. A sample was considered to have evidence of Zika virus neutralizing antibody if the Zika virus PRNT<sub>50</sub> titer was  $\geq 20$  and DENV-1 and DENV-2 FRNT<sub>50</sub> titers were  $< 20$  (2 samples) or if the Zika virus PRNT<sub>50</sub> titer was  $\geq 20$  and 4-fold greater than the DENV-1 and DENV-2 FRNT<sub>50</sub> titers (1 sample). Only 3 of 6 samples fulfilled these criteria; the remaining 3 contained detectable Zika virus, DENV-1, and DENV-2 antibodies, indicating past flavivirus infection of an indeterminate type. Thus, 3 (1.3%) of 234 samples were Zika virus seropositive, although we did not test for other flaviviruses.

The 3 Zika virus–seropositive monkeys were captured 35 km away (in Bukit Serendah, Selangor), 77 km away (in Kuala Lipis, Pahang), and 164 km away (in Manong, Perak) from Bentong (Pahang), where Zika virus was first isolated outside of Africa in 1966 (5). Of note, 5 of 6 samples with detectable Zika virus antibodies were collected in 2016, when human Zika virus cases were occurring in Malaysia and neighboring Thailand and Singapore. The rate of Zika virus antibody detection was higher in the 2016 collection (5.6%, 5/89) than the 2009–2010 collection (0.7%, 1/145;  $p = 0.031$  by Fisher exact test).

Our results indicate that wild long-tailed macaques in Peninsular Malaysia are exposed to Zika virus but at low levels, without evidence of viremia. This finding suggests that long-tailed macaques are unlikely involved in maintaining Zika virus sylvatic cycles in Malaysia, although the long-term dynamics of Zika virus antibodies and infection (including shedding) in macaques is unknown. This in-

**Table.** Zika virus, DENV-1, and DENV-2 neutralization titers of serum samples collected from long-tailed macaques in Peninsular Malaysia, 2009, 2010, and 2016\*

Sample collection period and size	No. samples†	Macaque sex and age group, ID no.	Town/City, state, coordinates	Neutralization titers			
				Zika virus PRNT <sub>50</sub>	Zika virus FRNT <sub>50</sub>	DENV-1 FRNT <sub>50</sub>	DENV-2 FRNT <sub>50</sub>
October–November 2009 and October 2010, $n = 145$	1	Male adult, ZMW604	Bukit Serendah, Selangor, 3.36°N, 101.60°E	640	640	<20	<20
March and August 2016, $n = 89$	5	Female juvenile, PMW804	Manong, Perak, 4.61°N, 100.90°E	40	20	<20	<20
		Female adult, WDSP/16/009	Kuala Lipis, Pahang, 4.18°N, 102.05°E	80	80	<20	20
		Male adult, WDSP/16/006	Kuala Lipis, Pahang, 4.18°N, 102.05°E	80	80	640	160
		Male adult, WDSP/16/012	Kuala Lipis, Pahang, 4.18°N, 102.05°E	40	40	20	40
		Male adult, Batu Pahat, Johor, 1.85°N, 102.94°E		40	20	40	20
		WDSP/16/086					

\*DENV-1, dengue virus serotype 1; DENV-2, dengue virus serotype 2; FRNT<sub>50</sub>, 50% focus reduction neutralization test; ID, identification; PRNT<sub>50</sub>, 50% plaque reduction neutralization test.

†Number of samples from the first batch ( $n = 234$ ) that were positive by Zika virus PRNT<sub>50</sub> and further tested by FRNT<sub>50</sub>.

formation is arguably needed before an animal can be designated a reservoir (8). Despite intense Zika outbreaks in humans, no active Zika virus infection and a low seroprevalence (2.9%) with low antibody titers was found in various NHP species in Brazil, suggesting that New World NHPs are unlikely to sustain sylvatic transmission cycles (9). Antibody responses after flavivirus infection are broadly cross-reactive and cross-neutralizing in the first few months after infection (10), but the effects against heterologous flaviviruses are poorly understood in wild macaques. Also, the circulation of Zika virus in macaques could be affected by the sylvatic cycles of other endemic flaviviruses. In conclusion, the low seroprevalence of Zika virus antibodies in long-tailed macaques reinforces the need to study other NHPs and mammals as reservoirs in Malaysia to elucidate Zika virus transmission and emergence.

### Acknowledgments

We thank the outbreak response team of the Wildlife Disease Surveillance Program, PERHILITAN, for collecting monkey samples.

This study was supported by a Malaysia One Health University Network research grant and the US Agency for International Development.

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## Severe Fever with Thrombocytopenia Syndrome Virus in Dogs, South Korea

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DOI: <https://doi.org/10.3201/eid2502.180859>

Of 103 serum samples collected from dogs in South Korea, 3 (2.9%) were positive for severe fever with thrombocytopenia syndrome virus (SFTSV) and 22 (21.4%) were positive for antibodies against SFTSV. A dog-derived isolate of SFTSV clustered with many South Korea SFTSV strains in the Japanese clade.

Severe fever with thrombocytopenia syndrome virus (SFTSV), a new tickborne phlebovirus of the *Phenuiviridae* family (previously *Bunyaviridae*), causes severe fever with thrombocytopenia syndrome (SFTS) in China, Japan, and the Republic of Korea (South Korea) (1). After identification of the first human case of SFTS in South Korea in 2013 (1), 335 cases (73 deaths; case-fatality rate 21.8%) were reported during 2013–2016 (2).

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## Appendix

### Cells and Viruses

C6/36 cells (*Aedes albopictus*, CRL-1660; American Tissue Culture Collection, <https://www.atcc.org/>) were maintained in Leibovitz's L-15 medium (Sigma-Aldrich, <https://www.sigmaaldrich.com/>) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Bovogen Biologicals, <https://bovogen.com/>) and 8% tryptose phosphate broth (Sigma-Aldrich). Vero cells (African green monkey kidney cells, 88020401; European Collection of Authenticated Cell Cultures, <https://www.phe-culturecollections.org.uk/>) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, <https://www.thermofisher.com/>) in the presence of 7.5% heat-inactivated FBS, 2 mM L-glutamine (Life Technologies), and 1 mM sodium pyruvate (Hyclone, <https://www.fishersci.com/>). Both media contained 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies). The C6/36 cells were incubated at 28°C in the absence of CO<sub>2</sub>, and Vero cells were incubated at 37°C in the presence of humidity and 5% CO<sub>2</sub>. C6/36 cells were used to propagate viruses, and Vero cells were used in virus titration and plaque reduction and focus reduction neutralization tests.

The Zika virus strain used in this study was MRS\_OPY\_Martinique\_PaRi\_2015 (European Virus Archive, <https://www.european-virus-archive.com/>). Dengue virus serotype 1 (DENV-1) Western Pacific and DENV-2 New Guinea C (provided by Shamala Devi Sekaran and Keivan Zandi, University Malaya, Kuala Lumpur, Malaysia) were propagated in C6/36 cells. The infectious virus supernatants were harvested after 5–7 days, precleared by centrifugation, aliquoted, and stored at –80°C. All viruses were titrated by focus immunoassay and stained with

pan flavivirus mouse monoclonal antibody D1–4G2–4-15 (4G2) (Absolute Antibody, <https://absoluteantibody.com/>). Plaque assays were performed to titrate Zika virus.

## **Virus Neutralization Assay**

Plaque reduction neutralization tests (PRNTs) were performed to detect the presence of Zika virus antibodies in monkey serum samples. A rapid screen for neutralizing antibodies against Zika virus was carried out. Heat-inactivated monkey serum samples were diluted in 1× Dulbecco's phosphate-buffered saline (PBS) at a 1:20 dilution and mixed with 100–120 PFU of Zika virus prediluted in DMEM 2% FBS to a final volume of 200 µL. The virus–antibody mixture was incubated for 1 hour at 37°C before inoculation with  $2.5 \times 10^5$  Vero cells in a well of a 24-well plate. The plate was further incubated for another hour at 37°C before replacing with plaque medium, 3 parts DMEM 3.5% FBS mixed with 2 parts 3% carboxymethylcellulose (Sigma-Aldrich). After 3 days incubation, the cells were fixed with 3.7% formaldehyde and stained with crystal violet. The number of plaques were enumerated, and serum samples that reduced the number of plaques >75% relative to virus control were selected and retested. The neutralizing titers of serum samples were expressed as PRNT<sub>50</sub>, the serum dilution that reduced plaque formation by 50%, and was determined with 2-fold serially diluted serum samples from 1:20 to 1:1,280.

Focus reduction neutralization tests (FRNTs) were carried out to confirm samples with Zika virus PRNT<sub>50</sub> titers  $\geq 20$  and to determine DENV-1 and DENV-2 antibody titers. A similar procedure was performed as described above, with 2-fold serially diluted serum dilutions from 1:20 to 1:1,280 mixed with 65–80 FFU of Zika virus, DENV-1, or DENV-2 except that methylcellulose (Sigma-Aldrich) was used in the plaque medium. After an incubation of 2 days (for Zika virus), 3 days (for DENV-1), and 5 days (for DENV-2), the plaque media was removed and the cells were rinsed with PBS before fixation with a chilled 1:1 mixture of methanol and acetone. The cells were blocked with 1% bovine serum albumin in PBS and incubated with 4G2 (250 ng/well) for 1 hour, followed by horseradish peroxidase–labeled goat anti-mouse IgG (Merck Millipore, <http://www.emdmillipore.com/>) at a 1:500 dilution for 1 hour. KPL TrueBlue Peroxidase substrate (SeraCare, <https://www.seracare.com/>) was used to visualize foci. Neutralizing titers were expressed as FRNT<sub>50</sub>, the serum dilution that reduced foci formation by

50% relative to virus control in the absence of serum. ZKA185, a neutralizing human monoclonal antibody (Absolute Antibody), served as the positive control for Zika virus neutralization assays and was used at a final concentration of 10 µg/mL.