

Zika Virus Seropositivity in 1–4-Year-Old Children, Indonesia, 2014

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We assessed Zika virus seroprevalence among healthy 1–4-year-old children using a serum sample collection assembled in 2014 representing 30 urban sites across Indonesia. Of 662 samples, 9.1% were Zika virus seropositive, suggesting widespread recent Zika virus transmission and immunity. Larger studies are needed to better determine endemicity in Indonesia.

Zika virus, first isolated in 1947 (1), is a flavivirus phylogenetically related to dengue virus (DENV) that is, like DENV, also transmitted by *Aedes* mosquitoes. Because of the epidemic that swept through the Americas in 2016, Zika virus infection is known to cause microcephaly, as well as other congenital defects and Guillain-Barré syndrome (2).

Zika virus has long been known to be endemic in Southeast Asia (3,4), but laboratory confirmation of infection can be challenging. Acute infections are often asymptomatic. In those who are symptomatic, viral RNA typically persists in blood <7 days and in urine <10 days after symptom onset, limiting the usefulness of nucleic acid testing (5). Zika virus antibody cross-reacting with DENV can confuse results of tests conducted in regions where the viruses co-circulate (6). Virus-specific neutralization assays can more accurately detect and measure Zika virus

antibody, but because of their complex requirements, these tests have seldom been used in epidemiologic studies (7).

Acute Zika virus cases have been reported in Indonesia (8), Singapore (9), Malaysia (10), Vietnam (11), and Thailand (12). However, little is known about Zika virus prevalence in the region. Limited retrospective testing of archived specimens collected from clinically ill patients in Thailand (12) and Cambodia (13) suggest that incidence in these countries is low. However, given the limited number of samples tested and lack of confirmatory testing in these studies, information on prevalence and distribution is challenging to assess. Likewise, little is known about the prevalence and geographic distribution of Zika virus in Indonesia, the biggest country in Southeast Asia.

DENV and chikungunya virus, also transmitted by *Aedes* mosquitoes, are endemic throughout Indonesia, suggesting the ecologic conditions exist for Zika virus transmission as well. An estimated 80% of the population in Indonesia is infected with ≥ 1 DENV by the age of 10 years (14). In our study, we assessed Zika virus seroprevalence among healthy 1–4-year-old children to determine the prevalence and distribution of Zika virus in Indonesia.

The Study

We used serum samples collected during October–November 2014 for a previous population-based, cross-sectional cluster survey conducted to assess DENV seroprevalence; in the study, 3,312 samples were collected from 1–18-year-old children in 30 urban districts in 14 provinces of Indonesia (14). In our study, we assessed only the children 1–4 years (range 12–59 months) of age because these children were least likely to have cross-reactive DENV antibodies. Ethics clearance was obtained from the Health Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia, and the US Centers for Disease Control and Prevention (CDC; Atlanta, Georgia, USA).

Plaque reduction neutralization tests (PRNTs) that could differentiate Zika virus neutralizing antibodies from those produced in response to DENV infection were adapted from protocols developed by the CDC (online Technical Appendix, <https://wwwnc.cdc.gov/EID/article/24/9/18-0582-Techapp1.pdf>). The challenge virus used in the PRNT was Zika virus JMB-185, acquired from a patient in 2014 (8). Convalescent serum from this same patient was used as a PRNT positive control. We subjected all specimens

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to 2 tiers of testing by PRNT₉₀ (i.e., a PRNT in which serum samples suppressing $\geq 90\%$ of challenge virus were considered positive for neutralizing antibody). In the first tier, we tested serum samples diluted 1:10. Samples that suppressed $\geq 90\%$ of Zika virus PFUs were considered potentially positive for Zika virus antibodies because DENV-specific antibodies, if present, could have cross-reacted and neutralized Zika virus. We then subjected the specimens considered potentially positive to a second PRNT₉₀, in which we tested serum samples against Zika virus and all 4 DENV serotypes (online Technical Appendix). Specimens that tested positive for Zika virus neutralizing antibody and negative for DENV neutralizing antibody by PRNT₉₀ were classified as Zika virus seropositive, as were specimens that had Zika virus PRNT₉₀ titers ≥ 4 -fold higher than all DENV PRNT₉₀ titers. We categorized specimens as flavivirus seropositive when Zika virus neutralizing antibodies were present but at titers < 4 -fold higher than any DENV neutralizing antibody titer (online Technical Appendix Table). We also tested a subset of samples for Japanese encephalitis virus antibody by PRNT₉₀; none of the samples tested had a titer > 20 , and none of the sample classifications were changed after testing.

In the initial PRNT₉₀ screening, we detected possible Zika virus antibody in 73 (11.0%) of the 662 serum samples (Table). Of these, 72 had a sufficient volume to undergo second-tier testing; 60 (83.3%) of 72 samples were Zika virus seropositive, and 12 (16.7%) were flavivirus seropositive. Serum samples from 11 of 14 provinces were Zika virus seropositive, and the collections from the provinces ranged from $\approx 4.5\%$ seropositive (North Sumatra, Banten, East Kalimantan) to $> 18\%$ seropositive (Central Java, Jambi; Figure). Overall, Zika virus seroprevalence in the 1–4-year-old cohort was 9.1% (95% CI 3.95%–11.01%).

Our assessment, involving use of the PRNT₉₀, which is highly specific for Zika virus antibodies, indicates widespread, recent Zika virus infection in much of western and central Indonesia. Our criterion for confirmed Zika virus antibodies (i.e., PRNT₉₀ titer for Zika virus ≥ 4 -fold higher than that for any DENV in the same specimen) is the international standard. In just 2% (12/662) of specimens, we could not determine whether the antibodies were Zika virus or DENV specific. When using the more conservative criterion of only classifying a sample as positive for Zika virus antibodies if no DENV-specific neutralizing antibodies are detected, the number of Zika virus antibody-positive samples decreases by only 6, leaving 54 samples still classified as Zika virus seropositive. Further evidence for the validity of the PRNT₉₀ was that DENV neutralizing antibody-positive samples were negative for the presence of Zika virus neutralizing antibodies across a range of titers (R.T. Sasmono, unpub. data).

Although our data provide some evidence regarding geographic distribution, no information is presented regarding a specific threshold titer associated with clinical illness or a correlation between geography and titer. Further studies involving larger sample sets would be necessary to address these topics. The single age group, relatively small number of specimens, and limited number of sites, particularly from eastern parts of the country, do not give a comprehensive picture of endemicity throughout Indonesia. The small numbers of specimens available from most localities did not enable accurate estimation of the proportional differences between localities. We could perform PRNT₉₀ with samples from the remaining cohort (the 5–18-year-olds), but we expect higher percentages of non-specific flavivirus seropositivity in the samples from this older age group.

Table. Seropositivity of 1–4-year-old urban children for Zika virus and other flaviviruses, by province, Indonesia, October–November 2014*

Province	Serologic status, % (no. positive samples/total samples)		
	Suspected Zika virus seropositive†	Confirmed Zika virus seropositive‡	Flavivirus seropositive§
Aceh	0 (0/22)	0 (0/22)	0 (0/22)
North Sumatra	9.1 (2/22)	4.5 (1/22)	4.5 (1/22)
West Sumatra	18.2 (4/22)	13.6 (3/22)	4.5 (1/22)
Jambi	18.2 (4/22)	18.2 (4/22)	0 (0/22)
Lampung	8.7 (2/23)	8.7 (2/23)	0 (0/23)
Banten	4.4 (2/45)	4.4 (2/45)	0 (0/45)
DKI Jakarta	10.6 (7/66)	10.6 (7/66)	0 (0/66)
West Java	11.1 (17/153)	8.5 (13/153)	2.0 (3/153)
Central Java	20.5 (18/88)	18.2 (16/88)	2.3 (2/88)
East Java	11.7 (13/111)	9.0 (10/111)	2.7 (3/111)
Bali	0 (0/22)	0 (0/22)	0 (0/22)
East Kalimantan	4.5 (1/22)	4.5 (1/22)	0 (0/22)
South Sulawesi	0 (0/22)	0 (0/22)	0 (0/22)
Southeast Sulawesi	13.6 (3/22)	4.5 (1/22)	9.1 (2/22)
All provinces	11.0 (73/662), 95% CI 5.34–13.32	9.1 (60/662), 95% CI 3.95–11.01	1.8 (12/662), 95% CI 0.23–3.35

*DENV, dengue virus; PRNT₉₀, plaque reduction neutralization test with neutralization defined as $\geq 90\%$ reduction in challenge virus PFUs.

†Serum samples that neutralized $\geq 90\%$ of the challenge virus at a 1:10 dilution on initial Zika virus PRNT₉₀ screening.

‡Serum samples that neutralized Zika virus only or had a PRNT₉₀ titer ≥ 4 -fold higher for Zika virus than for any DENV.

§Serum samples that neutralized Zika virus and DENV and had a PRNT₉₀ titer for Zika virus that was < 4 -fold higher than that for any DENV.

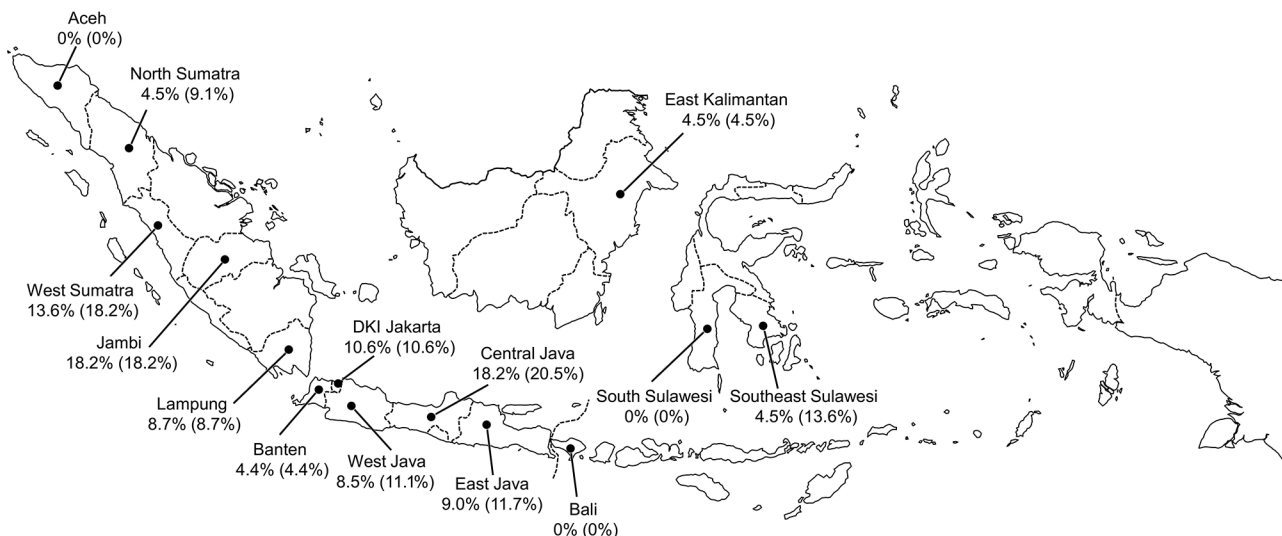


Figure. Geographic distribution of Zika virus–seropositive 1–4-year-old children, Indonesia, October–November, 2014. The values listed for each province indicate the percentage of serum samples confirmed Zika virus seropositive (percentage serum samples suspected to be Zika virus seropositive). Samples suspected to be Zika virus positive were those that were positive on initial Zika virus PRNT₉₀ (plaque reduction neutralization test with neutralization defined as $\geq 90\%$ reduction in challenge virus PFUs) screening when using a 1:10 serum sample dilution. Serum samples confirmed as Zika virus seropositive were those that neutralized Zika virus only or had a PRNT₉₀ titer for Zika virus that was ≥ 4 -fold higher than the PRNT₉₀ titer for any DENV.

Conclusions

Much has been published on epidemic Zika virus, but little is known about the effect of Zika virus in endemic areas. Determining the prevalence of Zika virus in Indonesia can provide clues to its potential long-term public health significance in endemic settings. Mild or asymptomatic infection is common, and confusion with dengue during diagnosis probably accounts for how long Zika virus was unrecognized in Indonesia and other areas of Southeast Asia. Besides the need to better evaluate Zika virus incidence and distribution, a high priority for future investigations will be determining the extent of Zika virus–related birth defects. If, like other flaviviruses, a primary Zika virus infection results in lifelong immunity, infections during childhood could reduce a person's risk for infection later in life and thus the incidence of Zika virus–related birth defects. This knowledge provides clues for understanding future patterns of Zika virus transmission in the Americas.

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References

- Dick GWA, Kitchen SF, Haddow AJ. Zika virus. I. Isolations and serological specificity. *Trans R Soc Trop Med Hyg.* 1952;46:509–20. [http://dx.doi.org/10.1016/0035-9203\(52\)90042-4](http://dx.doi.org/10.1016/0035-9203(52)90042-4)
- World Health Organization. WHO statement on the first meeting of the International Health Regulations (2005) (IHR 2005) Emergency Committee on Zika virus and observed increase in neurological disorders and neonatal malformations. 2016 Feb 1 [cited 2018 Apr 4]. <http://www.who.int/mediacentre/news/statements/2016/1st-emergency-committee-zika/en/>
- Musso D, Gubler DJ. Zika virus. *Clin Microbiol Rev.* 2016;29:487–524. <http://dx.doi.org/10.1128/CMR.00072-15>
- Duong V, Dussart P, Buchy P. Zika virus in Asia. *Int J Infect Dis.* 2017;54:121–8. <http://dx.doi.org/10.1016/j.ijid.2016.11.420>
- Paz-Bailey G, Rosenberg ES, Doyle K, Munoz-Jordan J, Santiago GA, Klein L, et al. Persistence of Zika virus in body fluids—preliminary report. *N Engl J Med.* 2017;NEJMoa1613108. <http://dx.doi.org/10.1056/NEJMoa1613108>
- Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. *Emerg Infect Dis.* 2008;14:1232–9. <http://dx.doi.org/10.3201/eid1408.080287>
- Posen HJ, Keystone JS, Gubbay JB, Morris SK. Epidemiology of Zika virus, 1947–2007. *BMJ Glob Health.* 2016;1:e000087. <http://dx.doi.org/10.1136/bmjgh-2016-000087>
- Perkasa A, Yudhaputri F, Haryanto S, Hayati RF, Ma'roef CN, Antonjaya U, et al. Isolation of Zika virus from febrile patient, Indonesia. *Emerg Infect Dis.* 2016;22:924–5. <http://dx.doi.org/10.3201/eid2205.151915>

9. Ho ZJM, Hapuarachchi HC, Barkham T, Chow A, Ng LC, Lee JMV, et al.; Singapore Zika Study Group. Outbreak of Zika virus infection in Singapore: an epidemiological, entomological, virological, and clinical analysis. *Lancet Infect Dis.* 2017;17:813–21. [http://dx.doi.org/10.1016/S1473-3099\(17\)30249-9](http://dx.doi.org/10.1016/S1473-3099(17)30249-9)
10. Salehuddin AR, Haslan H, Mamikutty N, Zaidun NH, Azmi MF, Senin MM, et al. Zika virus infection and its emerging trends in Southeast Asia. *Asian Pac J Trop Med.* 2017;10:211–9. <http://dx.doi.org/10.1016/j.apjtm.2017.03.002>
11. Chu DT, Ngoc VTN, Tao Y. Zika virus infection in Vietnam: current epidemic, strain origin, spreading risk, and perspective. *Eur J Clin Microbiol Infect Dis.* 2017;36:2041–2. <http://dx.doi.org/10.1007/s10096-017-3030-8>
12. Buathong R, Hermann L, Thaisomboonsuk B, Rutvisuttinunt W, Klungthong C, Chinnawirotpisan P, et al. Detection of Zika virus infection in Thailand, 2012–2014. *Am J Trop Med Hyg.* 2015;93:380–3. <http://dx.doi.org/10.4269/ajtmh.15-0022>
13. Duong V, Ong S, Leang R, Huy R, Ly S, Mounier U, et al. Low circulation of Zika virus, Cambodia, 2007–2016. *Emerg Infect Dis.* 2017;23:296–9. <http://dx.doi.org/10.3201/eid2302.161432>
14. Prayitno A, Taurel A-F, Nealon J, Satari HI, Karyanti MR, Sekartini R, et al. Dengue seroprevalence and force of primary infection in a representative population of urban dwelling Indonesian children. [erratum in *PLoS Negl Trop Dis.* 2018;12:e0006467]. *PLoS Negl Trop Dis.* 2017;11:e0005621. <http://dx.doi.org/10.1371/journal.pntd.0005621>

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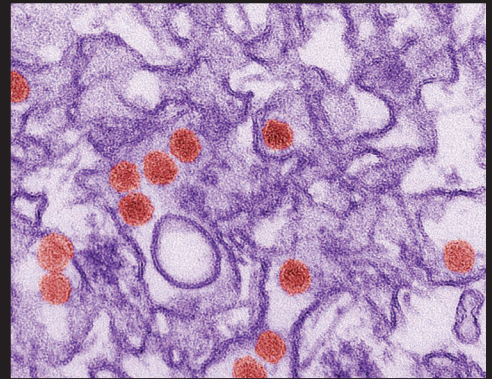


EID Podcast: Probable Unusual Transmission of Zika Virus

Zika virus (ZIKV), a mosquito-transmitted flavivirus, has been isolated from sentinel monkeys, mosquitoes, and sick persons in Africa and Southeast Asia. Serologic surveys indicate that ZIKV infections can be relatively common among persons in southeastern Senegal and other areas of Africa, but that ZIKV-associated disease may be underreported or misdiagnosed. In 2007, a large outbreak of ZIKV infection occurred on Yap Island in the southwestern Pacific that infected $\approx 70\%$ of the island's inhabitants, which highlighted this virus as an

emerging pathogen. The purpose of this study was to investigate and report 3 unusual cases of arboviral disease that occurred in Colorado in 2008.

Clinical and serologic evidence indicates that two American scientists contracted Zika virus infections while working in Senegal in 2008. One of the scientists transmitted this arbovirus to his wife after his return home. Direct contact is implicated as the transmission route, most likely as a sexually transmitted infection.



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Zika Virus Seropositivity in 1–4-Year-Old Children, Indonesia, 2014

Technical Appendix

Materials and Methods

Cell Lines

The BHK-21 cells (American Type Culture Collection, Manassas, VA, USA) used in plaque reduction neutralization tests (PRNTs) were grown and maintained in RPMI (Roswell Park Memorial Institute) medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA), and 1× antimicrobial-antimycotic drug solution (100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B; Thermo Fisher Scientific). The Vero cells (CCL-81, American Type Culture Collection) used for production of challenge viruses were grown in minimum essential medium supplemented with 5% FBS, 1× antimicrobial-antimycotic drug solution, and 2 mM L-glutamine. Cells were incubated in a controlled and humidified 37°C incubator with 5% CO₂ supplementation.

Serum Samples

Serum samples were derived from a dengue seroprevalence study involving 1–18-year-old urban children in Indonesia (1). Serum samples were heat-inactivated for 30 minutes at 56°C before use. A convalescent serum sample from the patient infected with Zika virus strain JMB-185 was used as the Zika virus–positive serum control. For the dengue virus (DENV) PRNTs, DENV–positive antibody controls were obtained from healthy volunteers who tested positive by DENV PRNTs. The Zika virus–negative antibody control was obtained from a healthy volunteer who had previously tested negative by Zika virus PRNT, and the DENV–negative antibody control was obtained from another healthy volunteer who had previously tested negative by DENV PRNT, as detailed below.

Challenge Viruses

The challenge virus used in the Zika virus PRNT was Zika virus strain JMB-185, isolated in Indonesia in 2014 as described previously (2). The complete genome of Zika virus JMB-185 has been reported, and on the basis of phylogenetic analysis, this isolate was classified as belonging to the Asian lineage (3). The DENV challenge viruses were the parental strains of the Sanofi Pasteur (Lyon, France) recombinant CYD vaccine viruses (4), namely DENV-1 strain PUO-359, DENV-2 strain PUO-218, DENV-3 strain PaH881/88, and DENV-4 strain 1228. The DENV strains were kindly shared by Sanofi Pasteur. The source of the DENV strains and their use in DENV neutralization assays have been described and accepted by the World Health Organization (5).

Challenge virus stocks were produced in Vero cells (seeded at 5×10^5 cells/flask 3 days before infection) by infecting cells at a multiplicity of infection (MOI) of 0.001 viruses/cell for Zika virus, DENV-1, DENV-2, and DENV-4, and an MOI of 0.01 viruses/cell was used for DENV-3. After the virus adsorption period of 90 minutes at 37°C with 5% CO₂, the inoculums were aspirated and replaced with fresh minimum essential medium containing 5% FBS, 1× antimicrobial-antimycotic drug solution, and 2 mM L-glutamine. Flasks were incubated in a 37°C incubator with 5% CO₂ supplementation until detectable signs of cytopathic effect (CPE) were seen on days 4–8. On the day of harvest, cell culture medium containing virus was collected and centrifuged for 10 minutes at $1,500 \times g$ and 4°C to remove cell debris. FBS and sorbitol (Sigma, St. Louis, MO, USA) were added to the clarified supernatant to a final concentration of 20% FBS and 10% (w/v) sorbitol to stabilize the virus. Virus stocks were aliquoted, flash-frozen, and transferred to a –80°C freezer for long-term storage.

Plaque Reduction Neutralization Test (PRNT)

The PRNT protocol was adapted from the US Centers for Disease Control and Prevention (Atlanta, Georgia, USA) and Sanofi Pasteur protocols (4,6,7). BHK-21 cells were seeded at 2.5×10^5 cells/well in 12-well tissue culture plates (Corning, Corning, NY, USA) and incubated for 2 days in a 37°C incubator with 5% CO₂. In each batch of samples tested, anti-Zika virus–positive and anti-Zika virus–negative serum samples were included as controls. Medium only was added to control wells. RPMI medium supplemented with 2% FBS, 2 mM L-glutamine, and 1×

antimicrobial-antimycotic drug solution was used as serum diluent for serum samples, controls, and challenge virus.

For initial Zika virus PRNT₉₀ (PRNT with neutralization defined as $\geq 90\%$ reduction in challenge virus PFUs) screening, unknown serum samples were diluted 1:5 in the wells of 96-well plates. A suspension of 60 PFUs of Zika virus strain JMB-185 in 60 μL was then mixed 1:1 with diluted serum samples and incubated for 1 h at 37°C to enable neutralization to occur. In addition, virus-only controls and two 10-fold serially diluted (1:10 and 1:100) virus-only controls were included to determine the PRNT₉₀ cutoff. After the neutralization step, culture medium was then aspirated from wells of 12-well plates containing BHK-21 cell monolayers. Without delay, the serum-virus suspensions were then inoculated onto designated wells of 12-well cell culture plates. Plates were incubated for 1 h at 37°C with agitation every 20 min to enable nonneutralized Zika virus to infect BHK-21 cell monolayers. At the end of virus adsorption period, the inoculum was then aspirated and each well was overlaid with 1 mL of 1% carboxymethylcellulose (CMC) RPMI medium supplemented with 2% FBS, 1 \times antimicrobial-antimycotic drug solution, 0.4% NaHCO₃, 2.5 mM HEPES, and 0.5% dimethyl sulfoxide. Plates were incubated at 37°C with 5% CO₂ for 5 days. After the incubation period, the CMC overlay medium was removed from wells and the cell monolayers were fixed with 3.7% formaldehyde solution for 30 minutes. Plates were washed with tap water and stained with 1% crystal violet staining solution for 5 minutes. Plates were finally rinsed with tap water and air-dried. The presence of Zika virus–infected cells was indicated by the formation of viral plaques, marked by a clear area of detached cells. Serum samples that neutralized $\geq 90\%$ of the challenge virus in the initial Zika virus screening were suspected Zika virus seropositive.

For the Zika virus–DENV PRNT₉₀ combination format with endpoint titrations, a similar assay setup was prepared, with the addition of DENV challenge viruses (DENV-1–4) and six 2-fold dilutions of the serum samples. Serum samples were initially diluted 1:5 in the first wells of 96-well plates followed by six 2-fold serial dilutions. Suspensions of Zika virus, DENV-1, DENV-2, DENV-3, and DENV-4 adjusted to 60 PFUs/60 μL were prepared. Diluted serum samples were mixed with an equal volume of each challenge virus, and after a 1-h neutralization step, the serum-virus suspensions were inoculated onto BHK-21 cell monolayers in 12-well plates. Inoculums were aspirated, and CMC medium was overlaid, followed by a 5-day

incubation period at 37°C with 5% CO₂. The cell monolayers were fixed with 3.7% formaldehyde solution and stained with 1% crystal violet staining solution.

The neutralization titer (PRNT₉₀ titer) of the serum samples was defined as the reciprocal of the highest test serum dilution for which the virus infectivity was reduced by 90% when compared with the average plaque count of the challenge virus controls. Plaques for all 6 serial dilutions of the serum samples were counted to ensure a dose-response reduction. The dilution factor of 2, generated by the addition of an equal volume of challenge virus to the diluted serum sample, was included in the final calculation of the neutralization titers (i.e., 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320). Hence, the theoretical lower limit of quantitation of the assay is a PRNT₉₀ titer of 10 (reciprocal of the dilution). Serum samples that neutralized Zika virus only or had a Zika virus PRNT₉₀ titer \geq 4-fold greater than the PRNT₉₀ titer of any DENV serotype were considered confirmed Zika virus seropositive. Serum samples that neutralized Zika virus and any DENV and had a Zika virus PRNT₉₀ titer $<$ 4-fold greater than the PRNT₉₀ titer of any DENV were considered flavivirus seropositive.

References

1. Prayitno A, Taurel A-F, Nealon J, Satari HI, Karyanti MR, Sekartini R, et al. Dengue seroprevalence and force of primary infection in a representative population of urban dwelling Indonesian children. *PLoS Negl Trop Dis*. 2017;11:e0005621. [PubMed](#)
<http://dx.doi.org/10.1371/journal.pntd.0005621>
2. Perkasa A, Yudhaputri F, Haryanto S, Hayati RF, Ma'roef CN, Antonjaya U, et al. Isolation of Zika virus from febrile patient, Indonesia. *Emerg Infect Dis*. 2016;22:924–5. [PubMed](#)
<http://dx.doi.org/10.3201/eid2205.151915>
3. Yudhaputri FA, Trimarsanto H, Perkasa A, Yohan B, Haryanto S, Wiyatno A, et al. Genomic characterization of Zika virus isolated from Indonesia. *Virology*. 2017;510:248–51. [PubMed](#)
<http://dx.doi.org/10.1016/j.virol.2017.07.026>
4. Timiryasova TM, Bonaparte MI, Luo P, Zedar R, Hu BT, Hildreth SW. Optimization and validation of a plaque reduction neutralization test for the detection of neutralizing antibodies to four serotypes of dengue virus used in support of dengue vaccine development. *Am J Trop Med Hyg*. 2013;88:962–70. [PubMed](#) <http://dx.doi.org/10.4269/ajtmh.12-0461>

5. Guirakhoo F, Arroyo J, Pugachev KV, Miller C, Zhang ZX, Weltzin R, et al. Construction, safety, and immunogenicity in nonhuman primates of a chimeric yellow fever-dengue virus tetravalent vaccine. *J Virol.* 2001;75:7290–304. [PubMed http://dx.doi.org/10.1128/JVI.75.16.7290-7304.2001](http://dx.doi.org/10.1128/JVI.75.16.7290-7304.2001)
6. Lindsey HS, Calisher CH, Mathews JH. Serum dilution neutralization test for California group virus identification and serology. *J Clin Microbiol.* 1976;4:503–10. [PubMed](http://pubmed.ncbi.nlm.nih.gov/1016811/)
7. Lindsey NP, Staples JE, Powell K, Rabe IB, Fischer M, Powers AM, et al. Ability to serologically confirm recent Zika virus infection in areas with varying past incidence of dengue virus infection in the United States and U.S. Territories in 2016. *J Clin Microbiol.* 2017;56:1–6. [PubMed http://dx.doi.org/10.1128/JCM.01115-17](http://dx.doi.org/10.1128/JCM.01115-17)

Technical Appendix Table. Endpoint titration results of combination Zika virus–DENV PRNT₉₀ of serum samples from 1–4-year-old children, Indonesia, October–November, 2014

No.	Sample ID	PRNT ₉₀ titer				Serostatus	
		Zika virus	DENV-1	DENV-2	DENV-3		DENV-4
1	02–1-08	80	<10	<10	<10	<10	Zika virus seropositive
2	02–1-10	40	<10	40	<10	<10	Flavivirus seropositive
3	03–1-03	80	<10	<10	<10	<10	Zika virus seropositive
4	03–1-08	160	<10	<10	<10	<10	Zika virus seropositive
5	03–1-12	80	<10	<10	<10	<10	Zika virus seropositive
6	03–1-13	40	<10	<10	<10	20	Flavivirus seropositive
7	04–1-06	80	<10	<10	20	<10	Zika virus seropositive
8	04–1-07	160	<10	<10	<10	<10	Zika virus seropositive
9	04–1-12	80	<10	<10	<10	<10	Zika virus seropositive
10	04–1-13	320	<10	<10	<10	<10	Zika virus seropositive
11	05–1-19	80	<10	<10	<10	<10	Zika virus seropositive
12	05–1-22	160	<10	<10	<10	<10	Zika virus seropositive
13	06–1-01	80	<10	20	<10	20	Zika virus seropositive
14	06–1-13	>320	<10	<10	10	<10	Zika virus seropositive
15	08–1-05	>320	<10	<10	<10	<10	Zika virus seropositive
16	09–1-22	>320	<10	10	20	<10	Zika virus seropositive
17	10–1-03	40	<10	<10	<10	<10	Zika virus seropositive
18	10–1-04	320	<10	<10	<10	<10	Zika virus seropositive
19	10–1-08	20	<10	<10	<10	<10	Zika virus seropositive
20	10–1-12	40	<10	<10	<10	<10	Zika virus seropositive
21	10–1-20	20	<10	<10	<10	<10	Zika virus seropositive
22	11–1-06	160	<10	<10	<10	<10	Zika virus seropositive
23	11–1-12	20	<10	<10	<10	<10	Zika virus seropositive
24	11–1-13	80	<10	<10	<10	<10	Zika virus seropositive
25	11–1-14	>320	<10	<10	<10	<10	Zika virus seropositive
26	11–1-16	40	<10	<10	<10	<10	Zika virus seropositive
27	11–1-22	20	20	10	80	<10	Flavivirus seropositive
28	12–1-01	20	<10	<10	<10	<10	Zika virus seropositive
29	12–1-19	20	<10	<10	<10	<10	Zika virus seropositive
30	12–1-21	40	<10	40	<10	<10	Flavivirus seropositive
31	13–1-01	40	20	<10	<10	<10	Flavivirus seropositive
32	13–1-10	160	<10	<10	<10	<10	Zika virus seropositive
33	13–1-14	80	<10	<10	<10	<10	Zika virus seropositive
34	13–1-22	160	<10	<10	<10	<10	Zika virus seropositive
35	14–1-12	160	<10	<10	<10	<10	Zika virus seropositive
36	16–1-12	160	<10	<10	<10	<10	Zika virus seropositive
37	16–1-21	80	<10	<10	<10	<10	Zika virus seropositive
38	18–1-02	80	<10	<10	<10	<10	Zika virus seropositive
39	18–1-06	160	<10	<10	<10	<10	Zika virus seropositive
40	19–1-01	160	<10	40	<10	<10	Zika virus seropositive
41	19–1-08	160	<10	<10	<10	<10	Zika virus seropositive
42	19–1-13	80	<10	<10	<10	<10	Zika virus seropositive
43	19–1-14	80	<10	<10	<10	<10	Zika virus seropositive

No.	Sample ID	PRNT ₉₀ titer				Serostatus	
		Zika virus	DENV-1	DENV-2	DENV-3		DENV-4
44	19-1-15	80	<10	<10	40	<10	Flavivirus seropositive
45	19-1-16	160	<10	<10	<10	<10	Zika virus seropositive
46	20-1-02	160	<10	<10	40	<10	Zika virus seropositive
47	20-1-04	160	>320	>320	20	<10	Flavivirus seropositive
48	20-1-07	40	<10	<10	<10	<10	Zika virus seropositive
49	20-1-11	80	<10	<10	<10	<10	Zika virus seropositive
50	20-1-13	20	<10	<10	<10	<10	Zika virus seropositive
51	21-1-02	320	<10	<10	<10	<10	Zika virus seropositive
52	21-1-03	80	<10	<10	<10	<10	Zika virus seropositive
53	21-1-06	20	<10	<10	<10	<10	Zika virus seropositive
54	21-1-07	>320	<10	20	<10	<10	Zika virus seropositive
55	21-1-10	320	<10	<10	10	<10	Zika virus seropositive
56	22-1-04	160	<10	<10	<10	<10	Zika virus seropositive
57	23-1-04	80	<10	<10	<10	<10	Zika virus seropositive
58	23-1-14	20	<10	<10	<10	<10	Zika virus seropositive
59	23-1-15	160	<10	<10	<10	<10	Zika virus seropositive
60	23-1-20	>320	<10	<10	<10	<10	Zika virus seropositive
61	23-1-22	320	<10	<10	20	<10	Zika virus seropositive
62	24-1-02	40	<10	<10	<10	<10	Zika virus seropositive
63	24-1-16	40	<10	<10	<10	<10	Zika virus seropositive
64	24-1-21	80	<10	<10	<10	<10	Zika virus seropositive
65	25-1-06	80	80	<10	<10	<10	Flavivirus seropositive
66	26-1-03	320	<10	<10	<10	<10	Zika virus seropositive
67	26-1-14	20	40	<10	<10	<10	Flavivirus seropositive
68	26-1-22	20	<10	80	<10	<10	Flavivirus seropositive
69	28-1-03	80	<10	<10	<10	40	Flavivirus seropositive
70	30-1-01	320	<10	<10	<10	<10	Zika virus seropositive
71	30-1-12	320	<10	<10	<10	<10	Zika virus seropositive
72	30-1-22	160	<10	<10	<10	160	Flavivirus seropositive

*DENV, dengue virus; ID, identification; PRNT₉₀, plaque reduction neutralization test with neutralization defined as an $\geq 90\%$ reduction in challenge virus PFUs.