

# Prevalence of Zika virus neutralizing antibodies in healthy adults in Vietnam during and after the Zika virus epidemic season: a longitudinal population-based survey

**Co Thach Nguyen**

Graduate School of Biomedical Sciences, Nagasaki University

**Meng Ling Moi** (✉ [sherry@nagasaki-u.ac.jp](mailto:sherry@nagasaki-u.ac.jp))

Institute of Tropical Medicine, Nagasaki University

**Thi Quynh Mai Le**

National Institute of Hygiene and Epidemiology

**Thi Thu Thuy Nguyen**

National Institute of Hygiene and Epidemiology

**Thi Bich Hau Vu**

National Institute of Hygiene and Epidemiology

**Hai Tuan Nguyen**

National Institute of Hygiene and Epidemiology

**Thi Thu Hang Pham**

National Institute of Hygiene and Epidemiology

**Thi Hien Thu Le**

National Institute of Hygiene and Epidemiology

**Le Manh Hung Nguyen**

Tay Nguyen Institute of Hygiene and Epidemiology

**Minh Huong Phu Ly**

Institute of Tropical Medicine, Nagasaki University

**Chris Fook Sheng Ng**

Program for Nurturing Global Leaders in Tropical and Emerging Communicable Diseases, Nagasaki University

**Taichiro Takemura**

Institute of Tropical Medicine, Nagasaki University

**Kouichi Morita**

Institute of Tropical Medicine, Nagasaki University

**Futoshi Hasebe**

Institute of Tropical Medicine, Nagasaki University

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## Research article

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## Abstract

**Background:** Between 2016 and 2019, 265 cases of Zika virus (ZIKV) infection were reported in Vietnam, predominantly in southern Vietnam. In 2016, a case of ZIKV-associated microcephaly was confirmed in the Central Highlands, and several members of the infant's family were confirmed to be infected with ZIKV. However, data on the seroprevalence of ZIKV antibodies in the general population is limited. The purpose of this survey was to determine the level of immunity to ZIKV in the general population of the ZIKV epidemic region.

**Methods:** A total of 879 serum samples were collected from 801 participants between January 2017 and July 2018, during and after the ZIKV epidemic in Vietnam. The samples were tested for anti-ZIKV immunoglobulin M (IgM) and immunoglobulin G (IgG), and anti-dengue virus (DENV) IgG antibodies using enzyme-linked immunosorbent assays (ELISA). Plaque-reduction neutralization test (PRNT) for ZIKV was performed on all samples, and for DENV on the samples that ZIKV neutralizing antibody positive.

**Results:** A total of 83 (10.3%) participants had anti-ZIKV IgM. Of the 83, 6 were confirmed to be ZIKV positive using PRNT and anti-ZIKV IgG ELISA. Of the 718 participants who were anti-ZIKV IgM negative, a further 3 cases were confirmed to be ZIKV positive. Of the 9 participants with ZIKV infection, 5 lived in the same village as the infant with ZIKV-associated microcephaly and the other 4 lived in 2 neighboring communes. Repeat samples were collected from the 83 ZIKV IgM positive participants 1.5 years after the first collection. No new cases of ZIKV infection were detected. In addition, 2 of 3 participants with anti-ZIKV NS1 IgG demonstrated a 4- to 8-fold increase in ZIKV neutralizing antibody titer.

**Conclusion:** ZIKV was present in the area around Krong Buk since at least 2016. While the low levels of circulation together with low seroprevalence suggests a limited outbreak in the region, the results also reflect on low levels of protective immunity to Zika within the population. These results provide a better understanding of the current ZIKV epidemic status in the region and demonstrate a need for implementation of more effective ZIKV infection control measures.

## Background

Zika virus (ZIKV) was first isolated from Rhesus monkeys in the Zika forest in Uganda in 1947 [1]. It is a mosquito-borne virus belonging to the genus *Flavivirus* and family *Flaviviridae* [2], which is spread from person to person mainly through the bite of infected *Aedes aegypti* and *Aedes albopictus* mosquitoes [3]. ZIKV can also be transmitted through sexual intercourse or body fluids [4]. Common symptoms are rash, fever, arthralgia, and conjunctivitis [5]. While ZIKV infection is sometimes associated with only mild symptoms, it can also lead to severe complications including Guillain-Barré syndrome [6].

The first reported ZIKV epidemic occurred in Yap Island, Federated States of Micronesia, in 2007, with an estimated 5,000 of a total of 6,800 residents infected [5]. The second reported epidemic occurred in French Polynesia in 2013 and 2014, with an estimated 28,000 people infected, comprising approximately 11% of the population [7, 8]. As many as 1.3 million people may have been infected in an epidemic in 14 states of Brazil in 2015 and 2016 [9]. During the epidemic period, there was an exponential increase in the number of cases of infants born with microcephaly suspected to be associated with ZIKV [9, 10]. According to a July 2019 WHO there has been evidence of ZIKV transmission in 87 countries and territories in the Americas, Africa, Southeast Asia, and the Western Pacific region [11].

While Southeast Asia has been known as a ZIKV endemic region for more than 60 years, large ZIKV epidemics has only been reported recently [12]. Although the virus has been first isolated from mosquitoes in Malaysia in 1966, the first human cases were only reported in 1977 [13]. In 2016, a total of 455 cases were confirmed in Singapore [14] and, in Thailand, 386 cases were reported in 29 out of 76 provinces from 2015 to 2017 [15]. During this period, cases of ZIKV infection were also reported in other Southeast Asia nations including Malaysia [16] and Myanmar [17]. In 2016, 3 tourists were confirmed to have ZIKV infection after visiting Vietnam [18–20]. As of June 2019, a total of 265 cases has been reported in Vietnam, most of which occurred in Ho Chi Minh City [21–24]. Additionally, in 2016, a case of Zika-associated microcephaly was reported in the Central Highlands of Vietnam and 5 family members and 2 neighbors were confirmed positive for ZIKV infection [25]. Despite the endemicity for dengue and the high density of mosquito vectors, the numbers of cases of ZIKV infection in Vietnam remain substantially lower than the number of cases of dengue. Vietnam, lies within the tropical zone where *Aedes aegypti* mosquitoes are endemic. While neighboring areas have reported ZIKV outbreaks in recent years, there are limited data available on the extent of ZIKV infection in local populations in Vietnam. Additionally, it has been hypothesized that dengue hyperendemicity may lead to cross-reactive immunity toward ZIKV, thus limiting the size of ZIKV epidemics in Southeast Asia, but there have been seroprevalence surveys to test this hypothesis. Cross-reactivity between ZIKV and DENV antibodies has led to difficulties in the interpretation in some studies [26]. Annually, Vietnam reports approximately 100,000 dengue cases. The incidence of dengue has remained stable over the past few decades in Vietnam [27], and dengue seroprevalence remains high with up to 64% of the adult population being seropositive [28]. Recent studies have suggested that while DENV is cross-reactive with ZIKV, the level of cross-neutralization and hence disease protection is limited [29–31]. In Vietnam, the number of cases ZIKV infection peaked at 219 in 2016 and subsequently decreased, with only one reported case in 2019 [32]. The purpose of this study was to determine

the seroprevalence of ZIKV antibodies among the population in Vietnam during and after the 2016 Zika epidemic using ZIKV and DENV neutralizing assays to determine the extent of the ZIKV epidemic in the local population.

## Methods

### Ethics approval and consent to participate

Ethics approval for this study was obtained from the ethics committee of the National Institute of Hygiene and Epidemiology, Ministry of Health, Vietnam (IRB-VN01057-45/2016) and the Ethics Committee of the Institute of Tropical Medicine, Nagasaki University, Japan (08061924-7).

### Sample collection

The samples were collected from participants by a simple random selection in Krong Buk District, Dak Lak Province where a case of ZIKV-associated microcephaly had been reported [25]. The population density in this region is 181 people/km<sup>2</sup> with the estimated total population of 65,000 living in an area of 357.82 km<sup>2</sup>. In January 2017, 3 months after the case of microcephaly was reported, blood samples were collected from healthy adults in the community with places of residence distributed across all communes in Krong Buk District. None of the study participants were hospitalized for an acute illness during the study period. In this study, we estimated the proportion of persons with ZIKV infection in Krong Buk District.

The required sample size was calculated assuming a precision/absolute error (d) of 4% and a proportion with a 95% level of confidence ( $Z_{1-\alpha/2} = 1.96$ ) [33]. Samples were processed within 24 hours of collection, and stored at  $-80^{\circ}\text{C}$  prior to testing.

### In-house Zika virus immunoglobulin M enzyme-linked immunosorbent assay

The samples were screened for ZIKV IgM using an in-house ZIKV IgM ELISA kit. This method was adapted from Dengue Virus IgM Capture DxSelect (Focus Diagnostics, Cypress, CA, USA) by replacing DENV antigen with ZIKV antigen [34]. Samples were diluted 1:100 using sample diluent solution. The 96-well IgM Capture plate (Dengue Virus IgM Capture DxSelect, Focus Diagnostics) were soaked with 1X wash buffer solution for 5 minutes, and wells were decanted. A total of 100  $\mu\text{L}$  of diluted serum sample was added and incubated at 37  $^{\circ}\text{C}$  for 1 hour. The plates were then washed 3 times with 1X wash buffer solution. Next, 100  $\mu\text{L}$  of ZIKV antigen (MR-766, 10<sup>5</sup> PFU/mL) was added and incubated at room temperature (RT) for 1 hour. The wells were then washed with 1X wash buffer solution for a total of 3 times to remove excess antigen. Next, 100  $\mu\text{L}$  of affinity-purified and peroxidase-conjugated mouse anti-flavivirus antibodies was added to all wells and the plate was incubated at RT for 30 minutes. The plates were washed 3 times to remove residual conjugate. Next, 100  $\mu\text{L}$  of tetramethylbenzidine (TMB) substrate solution and horseradish peroxidase was added to each well and the plate was then incubated at RT for 10 min in the dark. A total of 100  $\mu\text{L}$  of stop solution (1M sulfuric acid) was added to each well to stop the reaction. Finally, the plates were read at 450 nm of the optical density (OD) using an ELISA plate reader (Multiscan ELISA reader, ThermoLab System, Tokyo, Japan). OD values which were  $\geq 2$  times that of the negative control (N) is regarded as positive (P).

### Detection of dengue virus immunoglobulins M and G, and Zika virus immunoglobulin G by enzyme-linked immunosorbent assay

In addition to the detection of anti-ZIKV IgM antibodies by ELISA, anti-dengue IgM antibodies (Vircell, Granada, Spain) and anti-dengue IgG antibodies (Vircell) were determined according to manufacturer's instructions. Human Anti-Zika Virus IgG ELISA Kit (R&D Systems, Inc. Minneapolis, MN, USA) was used to test samples for ZIKV-specific NS1-antigen-reactive IgG antibodies in the samples that exhibited anti-ZIKV IgM antibodies and neutralizing antibodies to ZIKV according to manufacturer's instructions. OD values which were  $\geq 2$  times that of the negative control (N) were regarded as positive (P).

### Plaque-reduction neutralization test

Serum samples were screened for the presence of neutralizing antibodies to ZIKV using a plaque-reduction neutralization test (PRNT). Serum samples were inactivated at 56  $^{\circ}\text{C}$  for 30 minutes before testing. In the first PRNT screening for ZIKV, the serum was diluted 10 times in the EMEM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 2% FBS, then the serum samples were serially diluted 2-fold (1:10–1:10240). The PRNT was performed in replicates of 2 for ZIKV and all 4 DENV serotypes. At each dilution, a volume of 50  $\mu\text{L}$  serum was mixed with 50  $\mu\text{L}$  virus containing 100–200 plaque forming units (2000–4000 PFU/mL). The immune virus-complex mixture was then incubated at 37  $^{\circ}\text{C}$  for 1 hour. A total of 50  $\mu\text{L}$  of virus-immune complex mixture was then added onto BHK cell monolayers in 12 well plates (Corning Costar, Sigma-Aldrich, St. Louis, Missouri, USA) and incubated at 37  $^{\circ}\text{C}$  in 5% CO<sub>2</sub> for 1 hour. After incubation, overlay medium (2 mL of EMEM/ 1% methylcellulose (Wako Pure Chemical Industries Ltd, Osaka, Japan), in 2% FBS) was added into each well. The plates were

incubated at 37 °C in 5% CO<sub>2</sub> for 4–6 days until visible plaque formation. Cells were then fixed using 4% paraformaldehyde phosphate buffer solution (Wako) for 1 hour at RT and then stained with 1.25% crystal violet (Wako). The plaques were then counted by naked eye. The neutralization titers, PRNT<sub>50</sub> and PRNT<sub>90</sub>, were defined as the highest serum dilution which reduced the number of plaques by 50% and 90% respectively.

## Data analysis

Descriptive analyses were performed, using frequencies and percentages for categorical variables; and means and standard deviations (SDs) for continuous variables. Odds ratios (ORs) and the 95% confidence intervals (CIs) were used to estimate the relative probability of ZIKV infection for each group. Multiple logistic regression was used to assess the association of sex, ethnicity, and age group with ZIKV infection. For comparison in specific groups, chi-squared test, Fisher's exact test was used appropriately. The analyses were performed using Stata 14.1 (StataCorp LP, College Station, Texas, USA) with 5% level of significance and two-tailed p values. The map was created using QGIS software.

## Results

### Demographic characteristics of participants

The serological surveillance study was randomly selected in the community of Krong Buk District. Serum samples were collected from 801 of 65,000 residents (1.2%) in January 2017. The median age of the study participants is 33 years, of which the age group from 16–60 years was predominant of the 663 participants (82.7%). A total of 87 samples were collected from children under the age of 15 years (10.9%). The proportion of females in the study is more than 73%. In the ethnicity group analysis, 503 samples (62.8%) were collected from Kinh group which known as the majority group in Vietnam, the minority ethnic group Ede distributed in 290 samples (36.2%), and other minority ethnic groups had 8 participants (1%). The number of pregnant women who participated in this study was 66 (8.24%).

### Zika virus and dengue seroprevalence

Of the 801 samples tested, 83 (10.3%) were positive for anti-ZIKV IgM antibodies (Table 1) with an average P/N ratio of  $3.30 \pm 1.48$ . All of the participants who tested positive were asymptomatic at the time of sample collection. The prevalence of anti-ZIKV IgM antibodies varied significantly by age ( $p = 0.05$ ) and was highest in the 46–60 year age group (14.2%) (Fig. 1(a)). The seroprevalence of ZIKV IgM did not differ significantly according to sex or ethnicity. Only one of the 66 pregnant women (1.5%) in this study was positive for anti-ZIKV IgM antibodies.

Table 1  
Demographic characteristics of the study participants (N = 801)

Variables	n	ZIKV IgM positive (%)	Crude OR (95% CI)	P-value	DENV IgG positive (%) (n = 83)	ZIKV neutralizing antibodies (% positive)	
Age group (years)	≤15	87	11 (12.6)	0.05	4/11 (36.3)	2/87 (2.3)	
	16–30	295	19 (6.4)				0.48 (0.22–1.04)
	31–45	241	31 (12.8)				1.02 (0.49–2.13)
	46–60	127	18 (14.2)				1.14 (0.51–2.55)
	≥60	35	3 (8.5)				0.65 (0.17–2.48)
	NA <sup>†</sup>	16	1 (6.3)				
Sex	Female	590	59 (10)	0.57	32/59 (54.2)	7/590 (1.2)	
	Male	211	24 (11.4)				1.15 (0.7–1.91)
Ethnicity	Ede	290	25 (8.6)	0.16	15/25 (60.0)	3/290 (1.0)	
	Kinh	503	58 (11.5)				1.42 (0.87–2.33)
	Other <sup>‡</sup>	8	0				
Pregnancy	66	1 (1.5)			0/1 (0.0)	0/66 (0.0)	
CI, confidence interval; DENV, dengue virus; IgG, immunoglobulin G; IgM, immunoglobulin M; NA, not available; OR, odds ratio; PRNT, plaque reduction neutralization test; ZIKV, Zika virus							
† Participants with missing information on age were excluded from the multivariate logistic regression analysis							
‡ Participants whose ethnicity was categorized as “Other” were combined with the “Ede” ethnic group in the multivariate logistic regression analysis.							

All 83 participants with ZIKV anti-IgM antibodies were negative for dengue IgM on ELISA; however, 49 (59.0%) ZIKV IgM positive cases were detected as positive for Dengue IgG ELISA (Table 1). The distribution of the ZIKV IgM antibodies positive cases, as well as DENV IgG antibodies positive cases, was observed in all age groups in this study (Fig. 1(a)). All testing results showed the positive rates of the majority Kinh ethnic group higher than the minority Ede ethnic group (Fig. 1(b)).

## Neutralizing antibody levels to Zika virus and dengue virus serotypes 1–4

The samples were first screened to detect the presence of neutralizing antibodies to ZIKV from the 1:20 of serum dilution. Of the 801 samples, 8 demonstrated neutralizing antibodies to ZIKV (PRNT<sub>50</sub> = 1: 40 to 1: 640). Of the 8 samples, 3 (Z141a, Z153a, Z735a) exhibited ZIKV antibody titers that were > 4-fold higher than the antibody titers for all 4 DENV serotypes (PRNT<sub>50</sub> ZIKV = 1: 160 to 1: 640) (Table 2). The remaining 5 samples (Z120a, Z138a, Z140a, Z420a, Z476a) were classified as probable ZIKV infections (PRNT<sub>50</sub> ZIKV = 1: 40–1: 640).

Table 2

Anti-Zika virus immunoglobulin M and G levels of 13 participants with neutralizing antibodies to Zika virus epidemic (N = 879)

Sample ID	ELISA results			Neutralizing antibody titer (PRNT <sub>50</sub> )					Neutralizing antibody titer (PRNT <sub>90</sub> )				
	ZIKV IgM (P/N ratio)	DENV IgM (Index Value)	Anti-ZIKV NS1 IgG (P/N ratio)	ZIKV	DENV-1	DENV-2	DENV-3	DENV-4	ZIKV	DENV-1	DENV-2	DENV-3	DENV-4
During epidemic (January 2017)													
Z120a <sup>†</sup>	3.3	0.1	2.2	160	320	320	< 20	< 20	< 20	320	80	< 20	< 20
Z140a	2.3	0.3	4.7 <sup>†</sup>	80	40	320	< 20	< 20	< 20	< 20	40	< 20	< 20
Z141a	4.9	0.1	3.4	640	160	80	< 20	< 20	40	80	< 20	< 20	< 20
Z153a <sup>‡</sup>	2.8	0.3	3.2	160	< 20	< 20	< 20	< 20	40	< 20	< 20	< 20	< 20
Z47a6	4.7	0.5	43.9 <sup>†</sup>	640	320	1280	20	< 20	80	320	160	< 20	< 20
Z735a	2.8	0.6	8.1	320	< 80	< 80	< 80	< 80	80	< 80	< 80	< 80	< 80
Z147a <sup>†</sup>	1.6	ND	4.5	320	80	160	20	40	80	< 20	80	< 20	< 20
Z472a	1.5	ND	46.1	5120	160	160	80	40	640	80	40	80	< 20
Z606a	1.6	ND	10.6	1280	< 20	< 20	< 20	< 20	160	< 20	< 20	< 20	< 20
Z138a	4.6	0.6	0.9	40	320	80	< 20	< 20	< 20	160	< 20	< 20	< 20
Z420a <sup>‡</sup>	3.7	0.1	0.2	160	160	160	20	< 20	20	20	40	< 20	< 20
Z587a	1.2	ND	0.2	320	640	640	1280	160	80	320	320	640	20
Z591a	1.2	ND	0.6	640	640	640	1280	80	80	320	160	640	< 20
Post-epidemic (July 2018)													
Z120b <sup>†</sup>	ND	ND	2.3	80	640	160	1280	20	20	320	40	320	< 20
Z140b	ND	ND	5.2	640	< 20	160	< 20	20	20	< 20	80	< 20	< 20
Z141b	ND	ND	2.7	640	160	20	160	< 20	80	80	< 20	40	< 20
Z476b	ND	ND	4.7	2560	320	320	320	80	160	80	80	160	< 20
Z735b	ND	ND	3.5	80	320	80	320	80	< 20	320	20	160	20
Z138b	ND	ND	0.5	80	40	< 20	40	< 20	< 20	20	< 20	20	< 20
DENV, dengue virus; DENV1-4, dengue virus serotypes 1–4; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; IgM, immunoglobulin M; N, negative; ND, not detected; P, positive; PRNT <sub>50</sub> , plaque-reduction neutralization test with neutralization defined as ≥ 50% reduction in challenge virus plaque-forming units; PRNT <sub>90</sub> , plaque-reduction neutralization test with neutralization defined as ≥ 90% reduction in challenge virus plaque-forming units; ZIKV, Zika virus.													
Figures in bold indicate positive results. <sup>†</sup> ZIKV antibodies were determined using anti-ZIKV NS1 IgG ELISA (P/N ratio ≥ 2). <sup>‡</sup> Z153 and Z420 were not available during the post endemic collection.													

Consecutive samples were collected from 78 of the 83 participants with anti-ZIKV IgM antibodies collected from the same participants 18 months after the first sample collection. Repeat samples were not collected from 5 participants who were unavailable at the time of the repeat sample collection. Two (Z153 and Z420) of the 8 participants with ZIKV neutralizing antibodies in their initial sample did not have neutralizing antibodies detected in their second sample. Two participants (Z120 and Z735) experienced a 2- to 4-fold increase, and 2 participants (Z140 and Z476) experienced a 4- to 8-fold increase in their ZIKV neutralizing antibody titers (Table 2). Of the 83 participants with anti-ZIKV IgM antibodies, 5 (6.0%; Z140, Z141, Z153, Z476 and Z735) had ZIKV neutralizing antibody titers that were at least 4-fold greater than their antibody titers against the 4 DENV serotypes tested.

Of the 718 samples that were negative for anti-ZIKV IgM antibodies, 5 (Z147a, Z472a, Z606a, Z587a and Z591a) demonstrated neutralizing antibodies to ZIKV (PRNT<sub>50</sub> = 1:320–1:5120). Among the 5 samples, 2 samples (Z472a and Z606a) demonstrated a 4-fold or greater level of neutralizing antibodies to all 4 DENV serotypes (Table 2). In addition, by testing all 879 samples collected in both rounds using PRNT, 13 participants (1.6%, N = 801) had detectable ZIKV neutralizing antibodies. Overall, 7 participants (0.9%, Z140, Z141, Z153, Z476, Z735, Z472 and Z606) demonstrated ZIKV neutralizing antibody titers that were at least 4-fold higher than their antibody titers to all 4 DENV serotypes, whereas the other 6 participants (0.8%) demonstrated comparable levels of ZIKV and DENV neutralizing antibodies. Thus, the results suggest that the 7 participants had been exposed to ZIKV during the 2016 ZIKV epidemic.

## Anti-Zika virus NS1 immunoglobulin G levels in cases of probable Zika virus infection

Levels of anti-ZIKV NS1 IgG antibodies in the 83 samples that exhibited anti-ZIKV IgM antibodies (N = 83) and samples that demonstrated ZIKV neutralizing antibodies were determined using anti-Zika Virus NS1 IgG ELISA (R&D Systems). Anti-ZIKV NS1 IgG assays are useful for confirming ZIKV infection because they are highly specific and possess minimal cross-reactivity to other flaviviruses [35]. Sixteen out of 83 ZIKV IgM positive samples (19.3%) demonstrated ZIKV NS1 specific IgG antibodies, with a median of P/N ratio of  $2.7 \pm 10.3$  (data not shown). Among the samples had demonstrated neutralizing antibodies to ZIKV, 6 samples (Z120a, Z140a, Z141a, Z153a, Z476a and Z735a) were also positive for anti-ZIKV NS1 IgG by using ELISA. All of 3 samples (Z141a, Z153a and Z735a) that demonstrated a 4-fold or greater ratio of ZIKV neutralizing antibody titers to DENV antibody titers also demonstrated ZIKV NS1 IgG antibodies (P/N ratio = 3.1–8.1). These results confirm that these 3 participants were exposed to ZIKV infection. Three of the 5 samples with ZIKV neutralizing antibodies were also confirmed positive for anti-ZIKV NS1 antibodies. The Anti-ZIKV NS1 IgG levels in the second samples collected in July 2018 were comparable to the levels in the first samples.

Five samples (Z147, Z472, Z587, Z591 and Z606) that demonstrated ZIKV neutralizing antibodies (N = 718, ZIKV IgM negative samples) were also positive for anti-ZIKV NS1 IgG antibodies. Two samples (Z472 and Z606) were positive for both ZIKV neutralizing antibodies and ZIKV NS1 IgG (P/N ratio = 10.6 and 46.1 respectively). In addition, one sample (Z147) was also positive for anti-NS1 antibodies with a P/N ratio of 4.5. However, out of the 19 samples that were positive for anti-ZIKV NS1 IgG antibodies, only 9 (Z120, Z140, Z141, Z153, Z476, Z735, Z147, Z472 and Z606) exhibited neutralizing antibodies against ZIKV with PRNT<sub>50</sub> titers  $\geq 20$ .

In summary, out of 801 participants tested in this study, by using 2 methods Anti-NS1 IgG ELISA and PRNTs, 9 (1.12%, Z120, Z140, Z141, Z147, Z153, Z735, Z476, Z472 and Z606) were confirmed to be positive for ZIKV. Of these 9 participants, 5 (Z120, Z140, Z141, Z147 and Z153) lived in Cu Pong commune, the same village as the infant with ZIKV-associated microencephaly and whose family members were confirmed as positive for ZIKV in our previous study [25]. This study however excludes samples from our previous study [25]. The 4 remaining cases (Z472, Z476, Z606 and Z735) were recorded in 2 neighboring communes, two cases in Chu Kbo (1.7%, Z472 and Z476) and two other cases in Pong Drang village (0.99%) (Fig. 2).

## Discussion

In this study, we determined the anti-ZIKV IgM and IgG antibodies among 801 participants who were recruited during ZIKV epidemic in Vietnam. Of the 83 first samples that demonstrated ZIKV IgM antibodies, only 8 (1.0%) had ZIKV neutralizing antibodies (PRNT = 1:40 – 1:640) with 3 cases of ZIKV infection confirmed using PRNT<sub>50</sub>. While anti-IgM ZIKV antibodies as may have lower specificity than other ZIKV assays, the test is useful for identifying probable ZIKV cases [32, 36]. In further tests by using anti-ZIKV NS1 IgG test, 3 of the 5 probable cases were confirmed positive for anti-ZIKV NS1 IgG antibodies. By using both PRNT (ZIKV, DENV1-4) and anti-ZIKV NS1 IgG assays in all 83 participants that demonstrated cross-reactive ZIKV IgM antibodies, only 6 (7.2%) samples fulfilled the criteria of ZIKV PRNT ratio  $> 4$  and presence of ZIKV-specific NS1 cross-reactive antibodies, confirming that these 6 individuals had recently become infected with ZIKV. Using samples from the same participants, the levels of antibodies were determined 18 months after the first sample collection (post-ZIKV epidemic). Among these 6 confirmed cases, 3 samples that were only ZIKV NS1 IgG antibodies positive during the first collection also demonstrated high ZIKV neutralization titers 18 months later. None of the ZIKV seropositive participants had an international travel history, suggesting that local transmission in the area.

While the prevalence of anti-ZIKV IgM antibodies was highest in the 46–60 year age group, there were no significant discrepancy in anti-ZIKV IgM seropositivity rates across age groups, indicating that the risk of ZIKV infection is homologous across different age groups. In contrast, a high proportion of participants demonstrated DENV IgG antibodies, with seropositivity increasing with age [37–39]. These results are consistent with those of other studies, indicating association with longer exposure due to persistent DENV endemicity. While there were high levels of DENV seropositivity (49/83; 59.0%), indicating DENV exposure in the community, the overall seropositive rates for ZIKV remains low (9/801; 1.1%). While low ZIKV seroprevalence, a proxy of protection, indicates vulnerability of the population of the region to the ZIKV epidemic, the results also suggest that DENV may offer limited cross-protection against ZIKV.

Among the participants, there was no significant difference in ZIKV Ig M seroprevalence according to sex. The higher percentage of female participants in this study is due to socioeconomic factors in this region, as adult males have a higher tendency to travel for employment opportunities in urban areas. While statistically insignificant, the proportion of ZIKV IgM seropositivity in the Kinh ethnic group was higher (11.5%) than that of the minority Ede ethnic group (8.6%). This tendency may be due to the Kinh ethnic group possessing higher proficiency in the national language, and are thus, more likely to travel and had higher mobility as compared to other minority ethnic groups. This may reflect an increased risk of infectious disease exposure due to ethnic and socio-economical associated activity and behaviors. However, in the context of local socioeconomic development, rural poverty is still predominant, particularly in terms of access to medical care. These socioeconomic factors may further drive inter-city migrant workers to travel between larger cities and rural areas, in addition to low seroprevalence rate in the region, these factors may in turn lead to further Zika epidemic expansion during outbreaks.

Of the 718 samples that were negative for ZIKV IgM antibodies, 5 samples demonstrated high levels of ZIKV neutralizing antibodies with titers from 1: 320–1: 5120. Additionally, 3 samples were also positive for anti-ZIKV NS1 IgG, 2 of these 3 samples were confirmed by PRNTs with neutralizing antibody titers of 1:1280–1:5120. These results indicate local ZIKV transmission within the healthy community in the Central Highlands of Vietnam. Overall, 9/801 (1.1%) of the ZIKV confirmed case as positive for ZIKV had 5/200 (2.5%) cases collected in Cu Pong village, the same commune with the case of microcephaly cases that we reported in the previous study. In the vicinity at Chu Kbo and Pong Drang villages, only 2/119 (1.68%) and 2/202 (0.99%) positive cases were identified, respectively. This result indicates that the circulation of ZIKV in Central Vietnam is limited, in the period of 2 years since the confirmation of nationwide ZIKV outbreak in Vietnam. In comparison with previous reports in Indochina, the ZIKV seroprevalence found in this study is at comparable levels with those of Cambodia [40] and Laos [41]. The low ZIKV seroprevalence, a proxy of protection against the disease in the community, however indicates that the community is at risk of ZIKV epidemic.

## Conclusion

This study confirms ZIKV infection in the Central Highlands of Vietnam and suggests that ZIKV has been present in the province since at least 2016. The prevalence of ZIKV-specific antibodies was 1.1% at the start of the study period, suggesting a limited outbreak within the area. Economic factors including migrant workers may play a role in introducing emerging pathogens such as ZIKV to rural areas. While DENV seroprevalence remains high in the region, the overall low ZIKV seroprevalence and neutralizing antibody levels also indicates limited Zika disease protection in the population. Further studies of seroprevalence in the general population and continuous surveillance are needed to better understand the extent of the outbreak in the general population and to define the potential risk of ZIKV transmission in the region.

## Declarations

### Ethics approval and consent to participate

Ethics approval for this study was obtained from the ethics committee of the National Institute of Hygiene and Epidemiology, Ministry of Health, Vietnam (IRB-VN01057-45/2016) and the Ethics Committee of the Institute of Tropical Medicine, Nagasaki University, Japan (08061924-7). Written consent has been obtained from the study participants. For participants under the age of 16 years, written consent was obtained from parents or guardians.

### Consent for publication

Not applicable

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

CTN carried out the ELISA, PRNTs, performed data analyses, and drafted the manuscript. MLM, FH, and KM contributed reagents and materials, conceived the study, participated in study design and coordination, and drafted the manuscript. TQML, TTTN contributed reagents, and sample collection. TBHV, TTHP, THTL, TT contributed to screening samples by ELISA and molecular diagnosis. LMHN performed calculations in the sample size and sample collection. MHPL, CNFS, and HTN contributed to statistical analysis. All authors have read and approved the final manuscript.

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## List of abbreviations

DENV, dengue virus; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; IgM, immunoglobulin M; PRNT, plaque-reduction neutralization test; ZIKV, Zika virus

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## Figures

Figure 1a

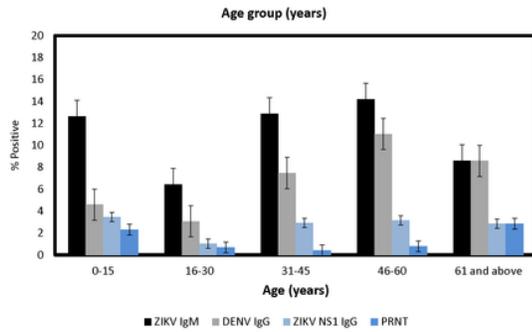


Figure 1b

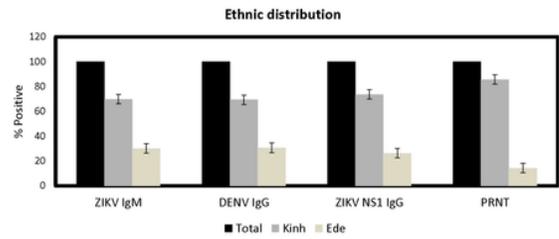


Figure 1

Seroprevalence of Zika virus antibodies in Central Vietnam, 2017-2018. The seroprevalence of Zika virus antibodies according to (a) age and (b) ethnicity. Anti-Zika virus antibodies were present in 83 participants, determined using enzyme-linked immunosorbent assays and were confirmed using Zika virus plaque-reduction neutralization test.

Figure 2

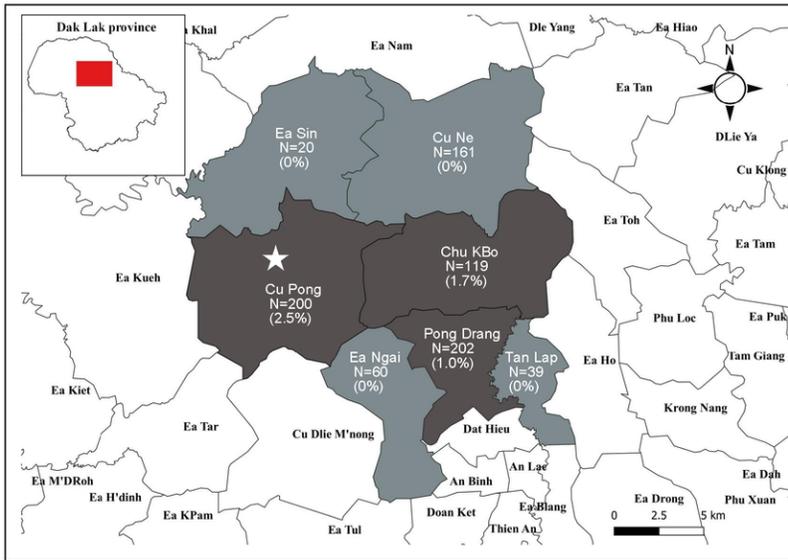


Figure 2

Zika virus antibody seroprevalence in the survey districts in Dak Lak Province in Central Vietnam. Zika virus seroprevalence was determined by testing serum samples of residents for Zika virus anti-NS1 antibodies using an enzyme-linked immunosorbent assay, and plaque-reduction neutralization tests. The dark gray shading indicates villages (local administrative units) in which Zika virus antibodies were found in one or more residents. The light gray shading indicates villages in which all study participants tested negative for Zika virus antibodies. N indicates the number of residents who were tested for Zika virus antibodies. The star denotes the village in which the Zika-associated microcephaly child was identified.