



รายงานวิจัยฉบับสมบูรณ์

โครงการ: การพัฒนาระบบ reverse genetics เพื่อการศึกษา
ระบาดวิทยาของไวรัส Zika

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มิถุนายน 2562

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สังกัด

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษาและ
สำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

1. บทคัดย่อภาษาไทย และภาษาอังกฤษ

บทคัดย่อ:

มีการรายงานการติดเชื้อไวรัสซิกาจากทั่วประเทศไทย แต่จำนวนผู้ป่วยที่รายงานยังคงต่ำ ซึ่งอาจจะบ่งชี้ถึงระดับการมีภูมิคุ้มกันป้องกันการติดเชื้อไวรัสซิกาในประชากรไทย เพื่อพิสูจน์ความเป็นไปได้นี้ จึงมีการตรวจแอนติบอดีชนิดลบล้างฤทธิ์ไวรัสซิกาในซีรัมจากผู้ใหญ่ชาวไทยที่มีสุขภาพดีจำนวน 135 คน ด้วยวิธี plaque reduction neutralization test (PRNT) โดยตรวจแอนติบอดีชนิดลบล้างฤทธิ์ต่อไวรัสเดงกีและไวรัสไข้สมองอักเสบเจอีด้วย ผลการทดสอบพบว่า 70.4% (50% PRNT หรือ $PRNT_{50} \geq 10$), 55.6 (PRNT₅₀ ≥ 20) or 22.2% (90% PRNT หรือ $PRNT_{90} \geq 20$) ของตัวอย่าง ตรวจพบแอนติบอดีชนิดลบล้างฤทธิ์ไวรัสซิกา การวิเคราะห์โดยละเอียดแสดงให้เห็นว่า ไม่มีความสัมพันธ์กันระหว่างการมีแอนติบอดีชนิดลบล้างฤทธิ์ต่อฟลาวิไวรัสอื่น ๆ (ไวรัสเดงกีและไวรัสไข้สมองอักเสบเจอี) กับการมีแอนติบอดีชนิดลบล้างฤทธิ์ไวรัสซิกา ผลการทดสอบนี้บ่งชี้ว่า แอนติบอดีชนิดลบล้างฤทธิ์ไวรัสซิกาในประชากรไทยเพียงพอที่จะรองรับการระบาดของไวรัสในประเทศไทย

Abstract

Zika virus (ZIKV) infections have been reported from all over Thailand, but the number of reported cases remains low, suggesting a degree of immune protection against ZIKV infection. To address this possibility, the presence of ZIKV neutralizing antibodies was determined in serum from 135 healthy Thai adults with a plaque reduction neutralization test (PRNT), and a number of samples were subsequently analyzed for the presence of neutralizing antibodies to dengue virus (DENV) and Japanese encephalitis virus (JEV). Results showed that 70.4% (PRNT₅₀ ≥ 10), 55.6 (PRNT₅₀ ≥ 20) or 22.2% (PRNT₉₀ ≥ 20) of the samples showed neutralizing antibodies to ZIKV. Detailed analysis showed no association between the presence of neutralizing antibodies to other flaviviruses (DENV, JEV) and the presence of ZIKV neutralizing antibodies. These results suggest that the level of ZIKV neutralizing antibodies in the Thai population is enough to dampen the transmission of the virus in Thailand.

2. Executive summary

Introduction

ZIKV was first isolated from a sentinel monkey in Zika forest Uganda in 1947 [1], and the virus was isolated from *Aedes africanus* mosquitoes in the same forest a year later [1]. The first reported human case of ZIKV infection was reported nearly a decade later, with infection again occurring in Zika forest in Uganda [2,3]. Between the initial identification of ZIKV and 2007 only a few sporadic cases of human ZIKV infection in Africa and Asia were reported (as reviewed elsewhere [4]). In 2007 a small outbreak on the islands of Yap State in Micronesia represented the first time the virus was detected outside of Africa and Asia and analysis suggested this virus has originated in Southeast Asia [5]. The virus again emerged from Southeast Asia in 2013 where it was detected as the causative agent in an outbreak of Zika fever in French Polynesia [6]. The virus subsequently spread to many of the islands in the Pacific Ocean, and was detected in Brazil in March 2015 [7,8], and within one year more than a million cases of infection were reported. From Brazil the virus spread quickly to other countries in South, Central and North America (as reviewed elsewhere [4]).

While serological studies have suggested the presence of ZIKV in Southeast Asia for more than 60 years [9–11], definitive evidence of the presence of the virus was first reported in 1966 [12], and the first virologically confirmed case of human infection was reported from Cambodia in 2010 [13]. In 2013 two tourists to Thailand were diagnosed on their return to their home countries with ZIKV infection [14,15] and a subsequent retrospective study by the Thai Ministry of Health confirmed that the virus was present in Thailand and causing disease in the local population [16], with the earliest confirmed cases dating to infections occurring in 2012. Currently, some 1,600 cases of ZIKV infection are reported to have occurred in 2016 and 2017 by the Thai Ministry of Public Health [17]. The basis of the markedly different population impact of ZIKV in the Americas and Southeast Asia remains unknown.

At least three human pathogenic mosquito transmitted flavivirus circulate in Thailand, and in addition to ZIKV, both dengue virus (DENV) and Japanese encephalitis virus (JEV) circulate. DENV consists of four closely related, but antigenically distinct viruses [18], termed DENV 1 to 4 all of which are endemic in Thailand [19]. Currently the main public health problem caused by these viruses is infections with DENV, as there are an average of 50,000 cases per year in Thailand, and DENV infection is the leading cause of hospitalization amongst children in Southeast Asia [20]. There is a national vaccination program to protect against JEV infections established in 1990 [21], however, JEV infection was the leading cause of hospitalizations for encephalitis in a study undertaken between 2003 and 2005 [22].

DENV infection induces protective immunity that lasts for many decades and possibly lifelong [23]. However, the immunity raised is against the homotypic virus only, and only

limited or transient protection is generated against heterotypic DENV, and thus multiple infections with DENV are possible [24]. Studies have shown that some 90% of Thai adults have antibodies against at least one DENV [25–27]. Given the national JEV vaccination campaign, adults and children below the age of around 30 are likely to have protective antibodies against JEV, while those older than that may not. The flaviviruses show high levels of homology [28], including in the main antigenic determinant the envelope (E) protein [29], and thus, as part of the immune response to an infection, cross reactive antibodies are raised [30]. While some studies have shown that anti-DENV antibodies can neutralize ZIKV [31,32], other studies have shown that anti-DENV antibodies may enhance ZIKV infection through the process of antibody dependent enhancement (ADE) of infection [33,34]. Mouse studies have suggested that protection or enhancement in ZIKV infection may depend upon the level of anti-DENV antibodies present [35]. However, non-human primate studies have suggested that preexisting immunity to DENV has no effect on ZIKV infection [36,37], and recent longitudinal studies on populations from Asia and the Americas have suggested that only low levels of cross neutralization of ZIKV are generated by prior DENV infection [38]. Nevertheless, it remains possible that the different population impact of ZIKV seen in Southeast Asia as compared to the Americas is a consequence of the existence of cross protection as a consequence of prior flaviviral infections and/or vaccination. This study sought to investigate the presence of ZIKV neutralizing antibodies in normal healthy Thais. In this respect the study is unique from other studies investigating ZIKV neutralizing antibodies that have mainly focused on the presence of these antibodies in patients with recent infections [39–42].

3. วัตถุประสงค์

Objective

To establish a plaque reduction neutralization test (PRNT) for application to diagnosis of Zika fever in Thailand

4. วิธีทดลอง

Materials and Methods

Blood sample collection and preparation

The study was performed in accordance with the Helsinki Declaration and was conducted after approval by the Mahidol University Central Institutional Review Board (Number: COA No. MU-CIRB 2017/067.2404). Written informed consent was received from all participants (cohort and controls). A total of 10 ml peripheral blood was collected into clot blood tubes (Greiner Bio One (Thailand) Ltd., Chonburi, Thailand) from the cohort of 135 healthy volunteers recruited through advertisement of the project at the Institute of Molecular Biosciences, Mahidol University during April–December 2017. There were 24 males and 111 females, with an age range of 18–63 years old. Whole blood samples were

placed at room temperature for 30 min. Two additional serum samples, one known ZIKV infection positive and one known flavivirus (DENV/JEV/ZIKV) negative were used as control samples. Serum was separated by centrifugation at 3,000 rpm for 10 min and kept at -20 °C until required.

Cells and viruses

Vero (ATCC Cat No. CCL-81) and LLC-MK2 (ATCC Cat No. CCL-7) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, Carlsbad, CA) supplemented with 5% heat inactivated fetal bovine serum (FBS; Gibco, Invitrogen) and 100 units/mL of penicillin/streptomycin (Gibco, Invitrogen). Cells were incubated at 37 °C with 5% CO₂. All viruses used in this study are shown in Supplemental Table S1. These viruses were propagated in C6/36 cells (ATCC Cat No. CRL-1660) with Minimum Essential Media (MEM; Gibco) supplemented with 10% FBS and 100 units/mL of penicillin/streptomycin. Virus supernatants were partially purified by centrifugation and supplemented with FBS up to 20%. All viruses were kept at -80 °C until required and their titers were quantitated on Vero cells or LLC-MK2 cells by the standard plaque assay as previously described [43].

Supplemental Table S1. Viruses used and their GenBank accession number

Virus	Virus Designation	Strain	GenBank Accession number
Zika virus SV0010/15	ZIKV SV0010	SV0010/15	KX051562.1
Japanese encephalitis virus	JEV	Beijing-1	U70389.1
Laboratory adapted dengue virus serotype 1	DENV 1 lab	16007	AF180818.1
Clinical isolated dengue virus serotype 1	DENV 1 DF	DENV-1/THAI/NS1-114/2006	KM519585
Laboratory adapted dengue virus serotype 2	DENV 2 lab	16681	M84727.1
Clinical isolated dengue virus serotype 2	DENV 2 DF	DENV-2/THAI/NS1-038/2006	KM519586
Laboratory adapted dengue virus serotype 3	DENV 3 lab	16562	KM519588
Clinical isolated dengue virus serotype 3	DENV 3 DF	DENV-3/THAI/NS1-007/2006	-a
Laboratory adapted dengue virus serotype 4	DENV 4 lab	1036	KM519590

Virus	Virus Designation	Strain	GenBank Accession number
Clinical isolated dengue virus serotype 4	DENV 4 DF	DENV-4/THAI/NS1-058/2006	KM519591

a No GenBank accession number is available (as reported in [49]).

Plaque Reduction Neutralization Test (PRNT)

Plaque reduction neutralization test (PRNT) of ZIKV and JEV was determined on Vero cells while PRNT of all DENV serotypes were determined on LLC-MK2 cells. Cells were seeded into 6-well plates at a density of 6×10^5 cells/well and grown at 37 °C for 24 h. Human serum was heat inactivated at 56 °C for 30 min. To detect neutralizing antibodies against ZIKV, all inactivated serum samples were initially diluted with BA-1 medium containing Medium 199 (Gibco, Invitrogen) and 10 mg/mL of bovine serum albumin fraction V at a dilution of 1:5 or 1:10 followed with serial two-fold dilutions. Some samples were diluted until the endpoint of neutralization activity was reached. Viruses were diluted with BA-1 medium to obtain a virus infectivity concentration of 600–2,000 PFU/mL (dependent upon virus). After that, 100 µl of prepared virus was incubated with an equal volume of each diluted serum or serum free BA-1 medium (non-neutralization control) in duplicate at 37 °C for 1 h. The virus and human serum mixture was inoculated onto confluent Vero or LLC-MK2 cells and incubated at 37 °C with constant agitation for 2 h. Inoculated cells were gently overlaid with first overlay solution containing 2X nutrient and 0.6% Seakem LE agarose (Cambrex, Bio Science Rockland Inc., Rockland, ME) for ZIKV or 1.6% for other viruses and maintained at 37 °C with 5% CO₂. On day six of incubation, cells were gently overlaid with a second overlay solution containing 2X nutrient, 0.5% neutral red and 1.6% Seakem LE agarose and incubated further for 15–17 h. Plaque number was counted and PRNT50 and PRNT90 are defined as a reciprocal of the highest dilution of tested serum that resulted in a reduction of viral infectivity by 50% or 90% when compared to the non-neutralization control, respectively. All PRNT results generated can be found in the supplemental data file.

Statistical analysis

The difference between flavivirus neutralizing antibody profiles in ZIKV PRNT90 < 20 and ZIKV PRNT90 \geq 20 cohorts was compared by independent sample t-test. A p-value of < 0.05 was considered significant. This analysis was performed using PASW statistics 18 (SPSS Inc., Chicago, IL).

3. ผลการทดลอง

Results and Discussion

Zika virus neutralizing antibodies in healthy Thai citizens

In a previous study, serum from 21 acute undifferentiated fever patients collected in Thailand was examined for immunoreactivity to ZIKV, DENV, JEV and CHIKV envelope proteins [44]. Immunoreactivity to at least one of the antigens was found in 20 of the 21 samples, with two samples showing immunoreactivity only to ZIKV E protein. However, a total of 14 of the samples showed immunoreactivity to ZIKV and at least one other antigen. This would suggest that ZIKV neutralizing antibodies are present at between approximately 10% (2/21) and 76% (16/21) of the Thai population. For this reason, a cohort size of 135 was selected which would be expected to have between 10 and 100 samples with neutralizing antibodies. Therefore, to study the presence of ZIKV neutralizing antibodies in healthy Thai adults, serum was collected from 135 healthy volunteers. The cohort was aged 18–63 years of age, consisting of 24 Thai males and 111 Thai females. Neutralizing antibodies were detected and quantitated by the gold standard for determining flavivirus neutralizing antibodies, the plaque reduction neutralization test (PRNT). PRNT of all human serum samples was initially determined against ZIKV strain SV0010/15 which was isolated from a Thai Zika fever patient [16] and represents the ZIKV currently circulating in Thailand. In an initial analysis, all samples were screened at a low level of stringency with ZIKV antibody positive samples being defined as those showing a PRNT₅₀ \geq 10. This cut-off value has been used in several recent studies determining levels of ZIKV neutralizing antibodies [39,45,46], as shown in Supplemental Table S2, and is additionally the default level recommended by the WHO in PRNT for DENV [47]. In this preliminary screen, samples were not subjected to assay of the endpoint of neutralization and only three conditions were screened (control, 10-fold dilution and 20-fold dilution). A total of 95 out of 135 samples (70.4%) showed PRNT₅₀ \geq 10 to ZIKV strain SV0010/15 (see Table 1 and Supplemental Table S3), while 73 out of the 135 samples (54.1%) showed a PRNT₅₀ \geq 20 (see Table 1 and Supplemental Table S3).

However, the WHO recommendation for DENV PRNT analysis suggest that in flavivirus endemic areas a PRNT₉₀ cutoff value is preferred [47], and several recent studies have used a 90% reduction in virus titer as the criteria for PRNT of ZIKV antibodies [40-42, 48]. However, these studies have variously used a 10-fold [40,42] or a 20-fold [41,48] initial dilution (see Supplemental Table S2). For greater stringency, this study used a 20-fold initial dilution, and thus positivity was defined as PRNT₉₀ \geq 20, and samples were further diluted to determine the endpoint of neutralization activity. At this level of analysis a total of 30 samples (22.2%) were positive for ZIKV neutralizing antibodies. (See Supplemental Table S4). We additionally recalculated the samples to find the number of samples that

would be positive with a 50% reduction of titer, and a total of 75 samples (55.6%) were positive for ZIKV antibodies at PRNT₅₀ \geq 20 (see Supplemental Table S5). This analysis was undertaken independently from the first screening analysis (which showed a PRNT₅₀ \geq 20 of 54.1%).

Supplemental Table S2. Summary of recent studies determining ZIKV PRNT and their starting dilutions and percent neutralization definition.

Title of study	Initial dilution (fold)	Positive % neutralization	Nomenclature	Reference
Imported arboviral infections in Italy, July 2014-October 2015: a National Reference Laboratory report.	10	50	PRNT ₅₀ \geq 10	[45]
Development of a Rapid Diagnostic Test Kit to Detect IgG/IgM Antibody against Zika Virus Using Monoclonal Antibodies to the Envelope and Non-structural Protein 1 of the Virus.	10	50	PRNT ₅₀ \geq 10	[39]
Mosquito Exposure and Chikungunya and Dengue Infection Among Travelers During the Chikungunya Outbreak in the Americas.	10	50	PRNT ₅₀ \geq 10	[46]
A Rapid Zika Diagnostic Assay to Measure Neutralizing Antibodies in Patients.	10	90	PRNT ₉₀ \geq 10	[42]
Development of Zika Virus Serological Testing Strategies in New York State.	10	90	PRNT ₉₀ \geq 10	[40]
Development and evaluation of a novel high-throughput image-based fluorescent neutralization test for	20	90	PRNT ₉₀ \geq 20	[48]

Title of study	Initial dilution (fold)	Positive % neutralization	Nomenclature	Reference
detection of Zika virus infection.				
Evaluation of Euroimmun Anti-Zika Virus IgM and IgG Enzyme-Linked Immunosorbent Assays for Zika Virus Serologic Testing.	20	90	PRNT ₉₀ ≥ 20	[41]

Table 1

Summary of plaque reduction neutralization test of 135 human serum samples against ZIKV strain SV0010/15.

PRNT50 value	Number of cases	Percentage (%)
<10	40	29.6
10	22	16.3
≥20	73	54.1
Total	135	100

Supplemental Table S3. ZIKV neutralizing antibodies at PRNT50 ≥ 10

PRNT50 of ZIKV strain SV0010/15 in 135 human serum samples (S1-S136).

PRNT50 ≥ 10 was defined as positive.

Sample	PRNT50	Sample	PRNT50	Sample	PRNT50	Sample	PRNT50
S1	10	S35	<10	S70	≥20	S104	≥20
S2	≥20	S36	≥20	S71	≥20	S105	10
S3	<10	S37	≥20	S72	≥20	S106	≥20
S4	≥20	S38	≥20	S73	≥20	S107	<10
S5	10	S39	≥20	S74	10	S108	≥20
S6	10	S40	≥20	S75	≥20	S109	≥20
S7	≥20	S41	≥20	S76	≥20	S110	≥20
S8	≥20	S42	≥20	S77	10	S111	≥20
S9	≥20	S43	≥20	S78	<10	S112	≥20
S10	<10	S44	<10	S79	<10	S113	≥20

Sample	PRNT50	Sample	PRNT50	Sample	PRNT50	Sample	PRNT50
S11	<10	S45	≥20	S80	≥20	S114	10
S12	<10	S46	10	S81	≥20	S115	<10
S13	<10	S47	10	S82	≥20	S116	≥20
S14	≥20	S48	≥20	S83	≥20	S117	<10
S15	<10	S49	10	S84	≥20	S118	10
S16	<10	S50	≥20	S85	<10	S119	≥20
S17	<10	S51	10	S86	≥20	S120	<10
S18	≥20	S52	10	S87	≥20	S121	≥20
S19	<10	S53	10	S88	<10	S122	<10
S20	10	S54	≥20	S89	≥20	S123	≥20
S21	≥20	S55	≥20	S90	≥20	S124	<10
S22	10	S56	<10	S91	<10	S125	<10
S23	10	S57	≥20	S92	≥20	S126	<10
S24	≥20	S58	<10	S93	<10	S127	≥20
S25	≥20	S59	<10	S94	≥20	S128	≥20
S26	10	S60	10	S95	≥20	S129	<10
S27	10	S61	≥20	S96	<10	S130	<10
S28	<10	S62	≥20	S97	≥20	S131	≥20
S29	<10	S64	<10	S98	≥20	S132	≥20
S30	≥20	S65	<10	S99	≥20	S133	<10
S31	≥20	S66	<10	S100	≥20	S134	≥20
S32	10	S67	<10	S101	≥20	S135	<10
S33	≥20	S68	≥20	S102	10	S136	≥20
S34	<10	S69	≥20	S103	≥20		

Supplemental Table S4. ZIKV neutralizing antibodies at PRNT90 \geq 20.

PRNT90 of ZIKV strain SV0010/15 in 135 human serum samples (S1-S136).

PRNT90 \geq 20 was defined as positive.

Sample	PRNT90	Sample	PRNT90	Sample	PRNT90	Sample	PRNT90
S1	<20	S35	<20	S70	40	S104	160
S2	<20	S36	80	S71	<20	S105	<20
S3	<20	S37	<20	S72	<20	S106	<20
S4	<20	S38	<20	S73	<20	S107	<20
S5	<20	S39	320	S74	<20	S108	<20
S6	<20	S40	160	S75	80	S109	<20
S7	<20	S41	160	S76	160	S110	<20
S8	<20	S42	<20	S77	<20	S111	<20
S9	160	S43	20	S78	<20	S112	320
S10	<20	S44	<20	S79	<20	S113	320
S11	<20	S45	<20	S80	<20	S114	<20
S12	<20	S46	<20	S81	320	S115	<20
S13	<20	S47	<20	S82	80	S116	<20
S14	<20	S48	<20	S83	<20	S117	<20
S15	<20	S49	<20	S84	20	S118	<20
S16	<20	S50	640	S85	<20	S119	80
S17	<20	S51	<20	S86	80	S120	<20
S18	<20	S52	<20	S87	<20	S121	<20
S19	<20	S53	<20	S88	<20	S122	<20
S20	<20	S54	<20	S89	160	S123	<20
S21	<20	S55	<20	S90	<20	S124	<20
S22	<20	S56	<20	S91	<20	S125	<20
S23	<20	S57	640	S92	20	S126	<20
S24	<20	S58	<20	S93	<20	S127	<20
S25	<20	S59	<20	S94	<20	S128	<20
S26	<20	S60	<20	S95	<20	S129	<20
S27	<20	S61	<20	S96	<20	S130	<20
S28	<20	S62	160	S97	<20	S131	20

Sample	PRNT90	Sample	PRNT90	Sample	PRNT90	Sample	PRNT90
S29	<20	S64	<20	S98	160	S132	320
S30	<20	S65	<20	S99	40	S133	<20
S31	<20	S66	<20	S100	160	S134	<20
S32	<20	S67	<20	S101	<20	S135	<20
S33	1,280	S68	160	S102	<20	S136	<20
S34	<20	S69	<20	S103	80		

Supplemental Table S5. ZIKV neutralizing antibodies at PRNT50 \geq 20

PRNT50 of ZIKV strain SV0010/15 in 135 human serum samples (S1-S136).

PRNT50 \geq 20* was defined as positive.

Sample	PRNT50	Sample	PRNT50	Sample	PRNT50	Sample	PRNT50
S1	<20	S35	<20	S70	160	S104	>320
S2	20	S36	>320	S71	40	S105	<20
S3	<20	S37	80	S72	40	S106	80
S4	20	S38	80	S73	<20	S107	<20
S5	<20	S39	>1,280	S74	<20	S108	<20
S6	20	S40	>320	S75	>320	S109	<20
S7	20	S41	>320	S76	>320	S110	<20
S8	20	S42	80	S77	<20	S111	<20
S9	640	S43	>320	S78	<20	S112	1,280
S10	<20	S44	20	S79	<20	S113	1,280
S11	<20	S45	40	S80	20	S114	20
S12	<20	S46	<20	S81	1,280	S115	<20
S13	<20	S47	<20	S82	320	S116	80
S14	20	S48	80	S83	80	S117	<20
S15	<20	S49	<20	S84	160	S118	20
S16	<20	S50	2,560	S85	<20	S119	160
S17	<20	S51	<20	S86	>320	S120	<20
S18	20	S52	<20	S87	40	S121	40
S19	<20	S53	<20	S88	<20	S122	<20
S20	80	S54	80	S89	>320	S123	40

Sample	PRNT50	Sample	PRNT50	Sample	PRNT50	Sample	PRNT50
S21	80	S55	40	S90	20	S124	<20
S22	40	S56	<20	S91	<20	S125	<20
S23	40	S57	>1,280	S92	40	S126	<20
S24	40	S58	<20	S93	<20	S127	160
S25	40	S59	<20	S94	<20	S128	20
S26	<20	S60	<20	S95	40	S129	<20
S27	20	S61	160	S96	<20	S130	<20
S28	<20	S62	>320	S97	20	S131	160
S29	<20	S64	<20	S98	>320	S132	2,560
S30	20	S65	<20	S99	160	S133	<20
S31	40	S66	<20	S100	>320	S134	40
S32	20	S67	<20	S101	<20	S135	<20
S33	>1,280	S68	>320	S102	<20	S136	80
S34	<20	S69	80	S103	>320		

* Samples were not titrated to limit of neutralization.

DENV, ZIKV and JEV neutralizing antibodies in healthy Thai citizens

It has been clearly established that anti-DENV antibodies can cross react with, and neutralize ZIKV [31,32]. For this reason, the neutralizing antibody profile to four laboratory adapted DENV serotypes (DENV 1–4 lab), four clinically isolated DENV serotypes (DENV 1–4 DF) as described previously [49] and JEV was evaluated for all 30 ZIKV neutralizing antibody positive samples (as defined by PRNT90 \geq 20), as well as a roughly sex and age matched equal number of ZIKV neutralizing antibody negative samples (Supplemental Tables S6 and S7). Samples were diluted to determine the endpoint of neutralization activity, and value of PRNT90 \geq 20 was defined as a positive result for all viruses. To confirm the validity of the PRNT assay, two further samples were screened. These were a 57 year old Caucasian resident of Thailand with no history of febrile illness, and a 22 year old female Thai with recent, clinically confirmed ZIKV infection. The Caucasian sample showed no evidence of neutralization for ZIKV, JEV or DENV, while the Thai sample showed a PRNT90 \geq 20 titer of 1,280 as well as neutralizing antibodies to JEV and DENV (Supplemental Table S8).

For the ZIKV neutralizing antibody positive samples (Table 2) two serum samples (S9 and S33) exhibited high levels of neutralizing antibodies to ZIKV but not to any of the DENVs or JEV. This result strongly suggests that these two serum samples carry specific

ZIKV neutralizing antibodies, raised as a consequence of ZIKV infection. Two further serum samples (S89 and S103) possessed neutralizing antibodies to ZIKV and JEV, but showed no evidence of DENV neutralizing antibodies, suggestive of ZIKV infection and JEV vaccination and/or infection. In Thailand, a mouse brain derived-inactivated JEV vaccine was initially included into the routine immunization schedule in 1990 [21] and it became a nationwide vaccination in 2000. Both S89 and S103 were 29 years of age at time of sample donation, and as such the JEV vaccination was possibly administered as part of the vaccination program, however detailed vaccination history of all participants was not collected. However, despite the presence of a national JEV vaccine program, cases of JEV encephalitis have been reported, even in vaccinated cases [22], so natural JEV infection cannot be ruled out. The rest of the samples showed complex profiles, with evidence of neutralizing antibodies to multiple DENV serotypes and/or JEV. Markedly however, the PRNT profiles for the ZIKV neutralizing antibody negative samples (Supplemental Table S9) were equally as complex. Markedly, if ZIKV neutralization was occurring as a consequence of cross-neutralizing antibodies, it would be expected that there would be a relationship between the degree of other flaviviral exposure and ZIKV neutralization. However, there was no association between ZIKV neutralizing antibody positivity and other flaviviral exposure, when DENV was treated as 4 flaviviral exposures (Supplemental Table S10) (p value = 0.102) or as one (Supplemental Table 11) (p value = 0.286) based on independent sample t-test. This suggests that where high titers of ZIKV neutralizing antibodies are found, they have arisen through past ZIKV infection, and as such would be consistent with the study by Montoya and colleagues [38].

While several studies have investigated ZIKV neutralizing antibodies [39,41,42,45,46,48], these studies have almost only been undertaken in the context of active ZIKV infection, and we believe this is the first study to determine the presence of ZIKV neutralizing antibodies in normal, healthy Thais. Interpretation of the results is somewhat contentious in light of the lack of a clearly defined correlation between absolute PRNT values and protective immunity. However, several studies have shown that a PRNT50 ≥ 10 is protective for Japanese encephalitis virus both in human [50–52] and animal [53] studies. Similarly, a PRNT50 ≥ 10 is defined as protective for yellow fever virus [54]. For DENV, the situation is more complex. While some studies report no correlation between PRNT and protective immunity [55], a second study assessed PRNT50 > 11 –16 being protective for DENV 1 and 4, but much higher PRNT values are required for protection against DENV 2 [56].

Supplemental Table S6. Gender of donors of samples screened for DENV and JEV PRNT by ZIKV PRNT status

Sample group	Gender		Total
	Male	Female	
ZIKV PRNT90 < 20	7	23	30
ZIKV PRNT90 ≥ 20	7	23	30

Supplemental Table S7. Age of donors of samples screened for DENV and JEV PRNT by ZIKV PRNT status

Age range	Sample group	
	ZIKV PRNT90 < 20	ZIKV PRNT90 ≥ 20
18-30	12	10
31-40	8	9
41-50	5	4
≥51	5	7
Total	30	30

Supplemental Table S8. Full screen of neutralizing antibody titer for ZIKV DENV (1-4) and JEV by plaque reduction neutralization test for two control samples (PRNT90 ≤ 20).

Sample	Gender	Age	ZIKV SV0010/15	JEV	DENV 1 lab	DENV 1 DF	DENV 2 lab	DENV 2 DF	DENV 3 lab	DENV 3 DF	DENV 4 lab	DENV 4 DF
S137	M	57	-	-	-	-	-	-	-	-	-	-
S63	F	22	1,280	640	160	160	320	160	160	20	160	320

Table 2

Full screen of neutralizing antibody titer for DENV (1–4) and JEV by plaque reduction neutralization test of ZIKV PRNT90 \geq 20 positive serum samples.

Sample	Gender	Age	ZIKV SV0010/15	JEV	DENV 1 lab	DENV 1 DF	DENV 2 lab	DENV 2 DF	DENV 3 lab	DENV 3 DF	DENV 4 lab	DENV 4 DF
S9	F	25	160	<20	<20	<20	<20	<20	<20	<20	<20	<20
S33	M	24	1,280	<20	<20	<20	<20	<20	<20	<20	<20	<20
S36	F	56	80	160	640	640	80	80	320	160	20	40
S39	F	47	320	<20	160	160	320	640	80	20	20	40
S40	F	53	160	80	80	80	20	20	40	20	<20	20
S41	F	50	160	80	160	160	80	20	40	80	<20	20
S43	F	49	20	320	320	640	320	320	160	80	80	160
S50	F	24	640	320	40	20	20	20	40	<20	40	80
S57	F	23	640	320	40	80	320	320	40	<20	<20	20
S62	F	37	160	<20	160	160	20	20	20	20	<20	20
S68	F	37	160	40	<20	20	<20	<20	<20	<20	40	20
S70	F	52	40	80	160	320	160	160	80	40	40	40
S75	M	37	80	<20	640	320	320	320	320	320	20	40
S76	F	36	160	<20	20	80	40	40	20	20	<20	<20
S81	F	52	320	<20	80	160	160	160	80	80	<20	20
S82	F	37	80	<20	80	80	40	40	40	20	<20	<20
S84	F	25	20	320	40	40	1,280	1,280	40	80	<20	20
S86	M	40	80	20	80	80	80	40	<20	<20	<20	<20
S89	F	29	160	160	<20	<20	<20	<20	<20	<20	<20	<20
S92	F	32	20	<20	160	320	320	320	80	40	40	40
S98	F	33	160	<20	80	160	320	160	80	20	40	160
S99	F	63	40	<20	20	40	20	20	40	40	20	40
S100	M	27	160	<20	320	160	160	40	80	40	<20	20
S103	F	29	80	80	<20	<20	<20	<20	<20	<20	<20	<20
S104	F	53	160	<20	80	160	80	40	40	<20	80	40

Sample	Gender	Age	ZIKV SV0010/15	JEV	DENV 1 lab	DENV 1 DF	DENV 2 lab	DENV 2 DF	DENV 3 lab	DENV 3 DF	DENV 4 lab	DENV 4 DF
S112	M	51	320	640	320	640	160	160	640	160	160	80
S113	M	37	320	<20	40	40	40	<20	20	<20	<20	<20
S119	F	43	80	<20	<20	40	<20	40	20	<20	<20	<20
S131	F	24	20	<20	20	80	20	<20	160	80	<20	<20
S132	M	27	320	80	20	<20	20	20	<20	<20	<20	<20

Supplemental Table S9. Full screen of neutralizing antibody titer for DENV (1-4) and JEV by plaque reduction neutralization test of ZIKV PRNT90 \geq 20 negative serum samples*.

Sample	Gender	Age	ZIKV SV0010/15	JEV	DENV 1 lab	DENV 1 DF	DENV 2 lab	DENV 2 DF	DENV 3 lab	DENV 3 DF	DENV 4 lab	DENV 4 DF
S7	F	25	<20	<20	320	160	320	160	160	80	20	80
S8	F	29	<20	20	80	40	320	160	80	80	<20	<20
S10	M	30	<20	<20	20	20	20	<20	80	40	<20	<20
S11	F	27	<20	40	320	640	40	40	40	20	<20	<20
S12	F	26	<20	40	<20	<20	<20	<20	<20	<20	<20	<20
S13	F	28	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
S14	F	28	<20	<20	<20	<20	320	160	<20	<20	20	20
S15	F	35	<20	<20	<20	20	<20	<20	80	40	<20	<20
S60	F	35	<20	20	80	80	320	160	40	20	<20	40
S64	F	36	<20	<20	<20	<20	80	160	<20	<20	40	40
S67	M	37	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
S77	F	52	<20	<20	40	160	20	<20	20	<20	40	40
S79	M	34	<20	20	40	160	80	80	320	320	80	40
S91	M	27	<20	40	<20	<20	<20	<20	<20	<20	<20	<20
S96	F	44	<20	<20	160	160	80	160	40	20	80	80
S101	M	20	<20	<20	40	80	<20	<20	640	320	<20	20

Sample	Gender	Age	ZIKV SV0010/15	JEV	DENV 1 lab	DENV 1 DF	DENV 2 lab	DENV 2 DF	DENV 3 lab	DENV 3 DF	DENV 4 lab	DENV 4 DF
S114	M	37	<20	20	20	40	80	160	<20	20	<20	20
S118	F	52	<20	<20	160	160	80	80	40	40	40	40
S120	F	41	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
S122	F	47	<20	<20	40	160	80	20	40	20	40	160
S123	F	54	<20	<20	320	640	160	80	160	160	20	20
S125	F	52	<20	<20	20	40	40	20	320	160	<20	20
S127	F	42	<20	20	160	160	320	160	40	20	20	40
S133	F	51	<20	20	<20	<20	<20	<20	<20	<20	<20	<20

*Plaque reduction neutralization test of selected flaviviruses in 30 serum samples that showed ZIKV PRNT90 < 20. The PRNT cutoff value at 90 (PRNT90) was defined as a reciprocal of the highest dilution of tested serum that resulted in the reduction of viral infectivity by 90%.

Supplemental Table S10. ZIKV PRNT90 positive and negative samples in relationship to other flavivirus PRNT results*.

Sample group	Number of flavivirus PRNT90 \geq 20						Total
	0	1	2	3	4	5	
ZIKV PRNT90 < 20	3	5	4	2	12	4	30
ZIKV PRNT90 \geq 20	2	2	0	8	9	9	30

4. สรุปและวิจารณ์ผลการทดลอง และข้อเสนอแนะสำหรับงานวิจัยในอนาคต

Overall, this study has shown that some 70% of serum samples examined had neutralizing antibodies against ZIKV (at PRNT50 \geq 10), a level that is protective for JEV [50–52] and YFV [54]. Modeling studies with DENV have suggested that population protection through herd immunity requires about 80% of the population to carry neutralizing antibodies [57]. While the figure of 70% seen here is lower than the predicted 80% figure, it is likely that such a level will significantly dampen the transmissibility of ZIKV, and possibly explains why, despite cases of ZIKV being detected over much of Thailand [17], the outbreaks are typically of small clusters that quickly die out.

5. ภาคผนวก

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1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่ได้คาดไว้ในสัญญาโครงการ

1. **Wikan, N.**, Smith, D. R. Zika virus from a Southeast Asian perspective. Asian Pacific journal of tropical medicine. 10(1):1-5, 2017. (Review article)
2. Sornjai, W., Jaratsittisin, J., Auewarakul, P., **Wikan, N.**, & Smith, D. R. Analysis of Zika virus neutralizing antibodies in normal healthy Thais. Scientific reports. 8(1):17193. 2018.

Asian Pacific Journal of Tropical Medicine 2017; 10(1): 1-5

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Review <http://dx.doi.org/10.1016/j.apjtm.2016.11.013>

Zika virus from a Southeast Asian perspective

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OPEN Analysis of Zika virus neutralizing antibodies in normal healthy Thais

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SCIENTIFIC REPORTS | (2018) 8:17193 | DOI:10.1038/s41598-018-35643-6

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