

Immunoglobulin M seroneutralization for improved confirmation of Japanese encephalitis virus infection in a flavivirus-endemic area

Tehmina Bharucha^{a,b,*}, Nazli Ayhan^c, Boris Pastorino^c, Sayaphet Rattanavong^b, Manivanh Vongsouvath^b, Mayfong Mayxay^{b,d,e}, Anisone Changthongthip^b, Onanong Sengvilaipaseuth^b, Ooyanong Phonemixay^b, Jean-David Pommier^{f,g,h,i}, Christopher Gorman^j, Nicole Zitzmann^a, Paul N. Newton^{b,e}, Xavier de Lamballerie^{ib,c}, and Audrey Dubot-Pérès^{b,c,e}

^aDepartment of Biochemistry, University of Oxford, Oxford, UK; ^bLao-Oxford-Mahosot Hospital-Wellcome Trust-Research Unit, Microbiology Laboratory, Mahosot Hospital, Vientiane, Lao PDR; ^cUnité des Virus Émergents, Aix-Marseille Univ-IRD 190-Inserm 1207, Marseille, France; ^dInstitute of Research and Education Development, University of Health Sciences, Ministry of Health, Vientiane, Lao PDR; ^eCentre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, UK; ^fEpidemiology and Public Health Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia; ^gInstitut Pasteur, Biology of Infection Unit, Paris, France; ^hInserm U1117, Paris, France; ⁱIntensive Care Department, University Hospital of Guadeloupe, France; ^jVirology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia

*Corresponding author: Tel: +01865275723; E-mail: t.bharucha@doctors.org.uk

Received 26 November 2021; revised 21 January 2022; editorial decision 21 February 2022; accepted 28 March 2022

Background: The mainstay of diagnostic confirmation of acute Japanese encephalitis (JE) involves detection of anti-JEV virus (JEV) immunoglobulin M (IgM) by enzyme-linked immunosorbent assay (ELISA). Limitations in the specificity of this test are increasingly apparent with the introduction of JEV vaccinations and the endemicity of other cross-reactive flaviviruses. Virus neutralization testing (VNT) is considered the gold standard, but it is challenging to implement and interpret. We performed a pilot study to assess IgG depletion prior to VNT for detection of anti-JEV IgM neutralizing antibodies (IgM-VNT) as compared with standard VNT.

Methods: We evaluated IgM-VNT in paired sera from anti-JEV IgM ELISA-positive patients (JE n=35) and negative controls of healthy flavivirus-naïve (n=10) as well as confirmed dengue (n=12) and Zika virus (n=4) patient sera. IgM-VNT was subsequently performed on single sera from additional JE patients (n=76).

Results: Anti-JEV IgG was detectable in admission serum of 58% of JE patients. The positive, negative and overall percentage agreement of IgM-VNT as compared with standard VNT was 100%. A total of 12/14 (86%) patient samples were unclassified by VNT and, with sufficient sample available for IgG depletion and IgG ELISA confirming depletion, were classified by IgM-VNT. IgM-VNT enabled JE case classification in 72/76 (95%) patients for whom only a single sample was available.

Conclusions: The novel approach has been readily adapted for high-throughput testing of single patient samples and it holds promise for incorporation into algorithms for use in reference centres.

Keywords: diagnostics, flavivirus, Laos, neglected tropical disease, neurological infection, seroneutralization

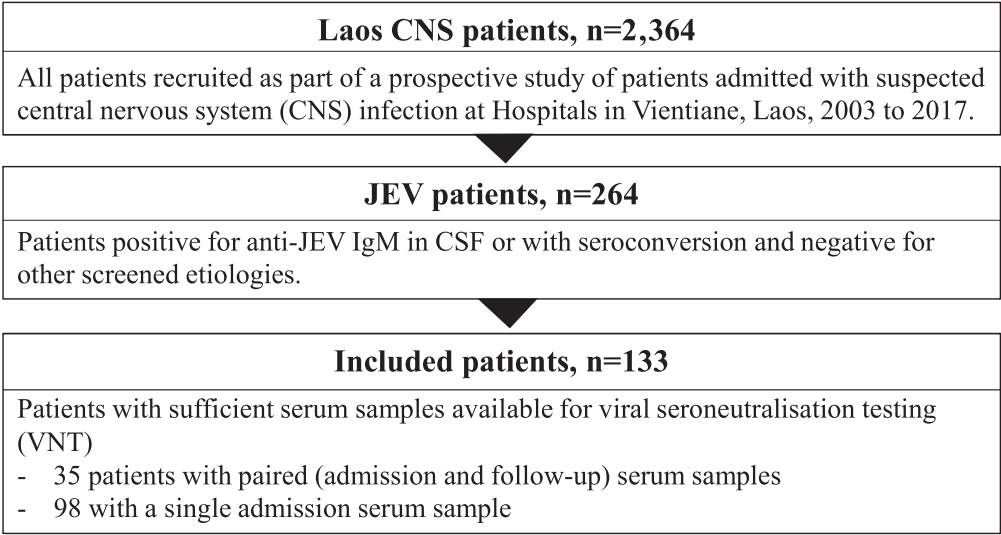
Introduction

Progress has been made in the implementation of vaccination programmes for Japanese encephalitis virus (JEV) in endemic areas.^{1–3} Nonetheless, gaps remain in understanding the epidemiology of the disease.^{2,4} Incorporation of JEV immunization in routine schedules and coverage remain suboptimal and there is inadequate surveillance to identify vaccine failure and JEV geographical expansion.^{2,5–8}

Detection of JEV nucleic acid is highly specific and provides additional molecular information.^{7,9} However, viraemia

is brief and low in humans and JEV RNA is rarely detected.¹⁰ Correspondingly, serological methods are the mainstay of diagnostic confirmation. The World Health Organization (WHO)-recommended test is the anti-JEV immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (JEV MAC-ELISA) to be performed and interpreted alongside an anti-dengue virus (DENV) MAC-ELISA.¹¹ The availability of commercial kits has facilitated widespread use of the JEV MAC-ELISA as the standard test. However, in line with other flaviviruses, there are increasingly recognized problems with specificity.^{12–15} For this reason, the

Patient selection



Patient sample analysis

Detection of anti-JEV IgG by ELISA, n=129	
156 admission +/- follow-up serum samples from 129 patients available (not enough volume after VNT for all samples). 102/156 (65%) samples with detectable IgG <ul style="list-style-type: none">- 72/125 (58%) admission serum- 30/31 (97%) of follow-up serum	

Patients with paired sera, n=35	Patients with single serum, n=98
Standard VNT, n=35	IgM VNT*, n=76**
VNT before IgG depletion <ul style="list-style-type: none">- 7 (20%) JE confirmed- 18 (51%) JE compatible- 10 (29%) Unknown	VNT after IgG depletion <ul style="list-style-type: none">- 63 (83%) JE confirmed- 3 (4%) JE compatible- 4 (5%) Unknown- 6 (8%) Negative
IgM VNT, n=18*	
VNT after IgG depletion <ul style="list-style-type: none">- 17 (89%) JE confirmed- 1 (5%) JE compatible- 1 (5%) Negative <p>* volume was not sufficient for full testing for 17 patients.</p>	<p>*VNT before IgG depletion was not performed due to insufficient sample volumes.</p> <p>**volume was not sufficient for full testing or IgG depletion was not successful, so results were not included in analysis, for 22 patients.</p>

Figure 1. Summary of the suspected JE patient samples tested.

Centers for Disease Control and Prevention (CDC) recommends that positive results obtained through JEV MAC-ELISA undergo confirmation by neutralizing antibody (NAb) testing.¹⁶ Gold-standard serological confirmation of JEV infection involves assessment of NAb titres using a virus neutralization test (VNT). This is more specific^{13,17} than the JEV MAC-ELISA. Conventional VNT methods involve a plaque reduction neutralisation

test (PRNT), however, laboratories are increasingly adopting high-throughput 96-well formats with comparable results.¹⁸ The high VNT requirements limit implementation: testing involves relatively large (>150 µL) sample volumes, the need for paired samples, biosafety 3 category laboratories, reference virus and cell strains and technical expertise. Indeed, interpreting VNT results is challenging due to cross-reactivity that is attributable

to anamnestic responses related to immunological reactions against a previously encountered flavivirus.¹⁹ As there are specific major overlaps in the distribution of JEV and other flaviviruses, contemporaneous VNT for other endemic flaviviruses is required. In Asia, this involves testing for DENV serotypes 1–4, Zika virus (ZIKV) and, in some areas, West Nile virus (WNV).²⁰ All of these viruses can manifest as neurological complications.²¹

Multiple methods have been attempted to mitigate cross-reactivity and anamnestic response interference in serological testing for non-JEV flaviviruses. These include analysis of IgA,^{22–31} IgG subclasses,²⁵ antibody avidity,^{22,32–35} incorporation of blocking agents^{34,36} and production of specific monoclonal antibodies for identification of specific viral epitopes.^{37–41} A modification of VNT, involving prior depletion of IgG, has been successfully performed for ZIKV¹⁹ and DENV infections.^{42,43} The underlying principle is that long-lasting IgG responses from vaccination and previous infection are major contributors to non-specific VNT results. IgG removal results in detection of specific neutralizing IgM antibodies, which are markers of acute infection.

We performed a pilot study to evaluate the utility of IgG depletion prior to VNT (IgM-VNT) to detect anti-JEV IgM neutralizing antibody for confirming acute JEV infection.

Methods

Patient samples

A prospective study of central nervous system (CNS) infections has been conducted at Mahosot Hospital, Vientiane, Laos, since 2003. Methods and results from 2003 to 2011 have been described.⁴⁴ Patients from 2014 to 2017 were included in the Southeast Asia Encephalitis Project.⁴⁵ The laboratory also receives samples from patients from other hospitals around Vientiane City (i.e. Friendship, Children's and Setthathirat Hospitals). Written informed consent was obtained from patients or responsible guardians. Anti-JEV and anti-DENV IgM were detected by the Japanese encephalitis/dengue IgM combo ELISA (Panbio, Brisbane, QLD, Australia; now Alere) until July 2014, for which result interpretation included a ratio between DENV and JEV. After August 2014, as per WHO recommendations, the JEV IgM ELISA (Inbios, Seattle, WA, USA) was utilized. All samples used were aliquoted and stored at -80°C . This pilot study involved a convenience sample of consecutive patients with available specimens to be tested; hence a sample size calculation was not performed.

Suspected JE patients included in this study had anti-JEV IgM detected by MAC-ELISA in cerebrospinal fluid (CSF) or seroconversion between acute and follow-up serum, no other pathogen detected in any body fluid and a sufficient volume of acute and/or follow-up serum for VNT. Patients with DENV and JEV RNA or DENV non-structural protein 1 (NS1) in serum or CSF were excluded.

Negative controls included samples from three groups: healthy flavivirus-naïve blood donors living in Puy-de-Dôme, in central France; ZIKV VNT-confirmed sera collected in Peru in the framework of a seroprevalence study⁴⁶; and DENV infection patients from the Laos CNS study (study details reported in

the section on suspected JE patients above), confirmed by IgM and/or NS1 ELISA and negative for anti-JEV IgM. All procedures relating to the conduct, evaluation and documentation of the study have been conceived in agreement with the good clinical practices and ethical principles of the Helsinki Declaration. Written informed consent was obtained from all subjects included in the study. All data and samples were anonymised.

Anti-JEV IgG ELISA

Anti-JEV IgG was detected using the Euroimmun ELISA kit (Lübeck, Germany) according to manufacturer's instructions. A standard curve using three calibration samples was used to calculate the concentration of antibodies in relative units (RU)/mL for each sample using optical density results; <16 RU/mL was negative, ≥ 16 – <22 RU/mL was equivocal and ≥ 22 RU/mL was positive.

IgG depletion

IgG depletion was performed using Protein G HP SpinTrap/Ab Spin Trap columns (28-4083-47; Cytiva, Marlborough, MA, USA). These contain recombinant protein G, a protein present in group G *Streptococcus* with high affinity for IgG. An in-house method developed by the French National Centre for Arboviruses was used, substituting commercial binding buffer by phosphate-buffered saline (PBS). Two IgG depletion columns were used for 100–150 μL sample serum. Columns were inverted three times and briefly vortexed. Each column was inserted in a 2-mL tube and centrifuged. All centrifugation steps were performed at 500 g for 2 min. The subsequent eluate was discarded, 600 μL of PBS added to each column and centrifuged again. Columns were transferred to clean 2-mL tubes and 100–150 μL of sample was added to one column and incubated at room temperature for 4 min before centrifugation. The eluate was transferred to the second column, incubated at room temperature for 4 min and centrifuged again. The final eluate was stored at -20°C until the VNT.

VNT

Two-fold dilutions from 1/20 to 1/2560 of each serum sample were tested in duplicate by VNT for JEV, DENV1–4, ZIKV and WNV. Serum dilutions from 1/10 to 1/1280 were prepared and mixed in a 1:1 ratio with 100 TCID₅₀ viral suspension (Table 1) using epMotion 5075 (Eppendorf, Hamburg, Germany) in a 96-well microplate (Figure S1). Negative controls containing minimum essential medium (MEM), with or without serum, were included in each microplate. Plates were incubated at 37°C for 2 h. A 100- μL suspension of Vero cells (ATCC CCL-81) containing approximately 2×10^5 cells/mL, was added to each well using the epMotion 5070 (Eppendorf) and incubated at 37°C in a 5% carbon dioxide incubator. After 5–7 d, microplates were read under an inverted microscope. Two investigators read the results for each replicate to identify the end dilution at which there was no cytopathic effect, with a

Table 1. Virus strain used in VNTs

Virus	Strain	Country of isolation	GenBank number	EVAg number	Titre (TCID ₅₀ /mL)	Day read
JEV	Laos 2009	Laos	KC196115	001V-02217	2 × 10 ⁹	5
WNV	UVE/WNV/2008/US/R94224	USA	–	001V-02224	2.1 × 10 ⁷	5
ZIKV	ZIKV strain H/PF/2013 French Polynesia	French Polynesia	KJ776791	–	3.7 × 10 ⁶	5
DENV-1	DENV1 2012	Saint Vincent and the Grenadines	VC16692	001V-02335	3.1 × 10 ⁷	7
DENV-2	UVE/DENV-2/1998/MQ/703	Martinique	AF208496	–	6.7 × 10 ⁴	5
DENV-3	UVE/DENV-3/2001/MQ/2023	Martinique	AH011666	–	4.5 × 10 ⁵	6
DENV-4	UVE/DENV-4/1998/ID/814	Indonesia	–	–	3 × 10 ⁶	6

EVAg: European Virus Archive – GLOBAL; TCID₅₀: 50% tissue culture infective dose.

third investigator to resolve disagreement. For duplicates, the geometric mean of end dilutions was calculated and reported as an NAb titre and ≥ 40 was considered as positive.^{47,48} Suspected JE patients were categorized as acute JE positive, confirmed or compatible, JE negative and unknown, according to the criteria in Figure 2.

Results

From 2003 to March 2021, 264 patients with suspected CNS infection were positive for anti-JEV IgM (in CSF or with seroconversion) and negative for other screened aetiologies⁴⁴ (see Figure 1). Paired serum samples (admission and follow-up) were available for 35 patients and a single acute sample for 98 patients. Among these 133 included patients, 130 (98%) had anti-JEV IgM detected in CSF and 3 (2%) demonstrated IgM seroconversion only (no anti-JEV IgM in CSF) in paired sera. The median age of the patients was 11 y (interquartile range [IQR] 6–20) and 32% (43/133) were female. The median duration of illness on admission was 5 d (IQR 4–6) and the median time between admission and follow-up serum collection was 14 d (IQR 10–25).

IgG depletion

A total of 102/156 (65%) serum samples, including 72/125 (58%) admission sera and 30/31 (97%) follow-up sera, were anti-JEV IgG positive by ELISA before IgG depletion. Seventy samples had sufficient volumes to be tested for anti-JEV IgG by ELISA after IgG depletion. Fifty-nine (84%) were negative or equivocal after IgG depletion. Six samples were equivocal before IgG depletion and all of these were negative after IgG depletion. Samples that remained positive after IgG depletion demonstrated decrease in the titre, however, the starting anti-JEV IgG result in these cases was high, all >125 RU/mL (positive >22 RU).

VNT for the patients with paired serum samples

VNT results prior to IgG depletion enabled classification of 25/35 (71%) patients as JE positive, 7 (20%) confirmed, 18 (51%)

compatible; and 10 (29%) as unknown (Table 2 and Table S2). Eighteen of these patients had sufficient serum available for IgM-VNT in at least one sample. The results enabled reclassification through the removal of cross-reactive IgG to other viruses and the specific detection of anti-JEV IgM, such that 17 (94%) were classified as JE positive, 16 (89%) confirmed, 1 (6%) compatible; and 1 (6%) as JE negative. Five patients classified as unknown by VNT did not have sufficient acute and/or follow-up sample to perform IgG depletion and/or anti-JEV IgG ELISA testing.

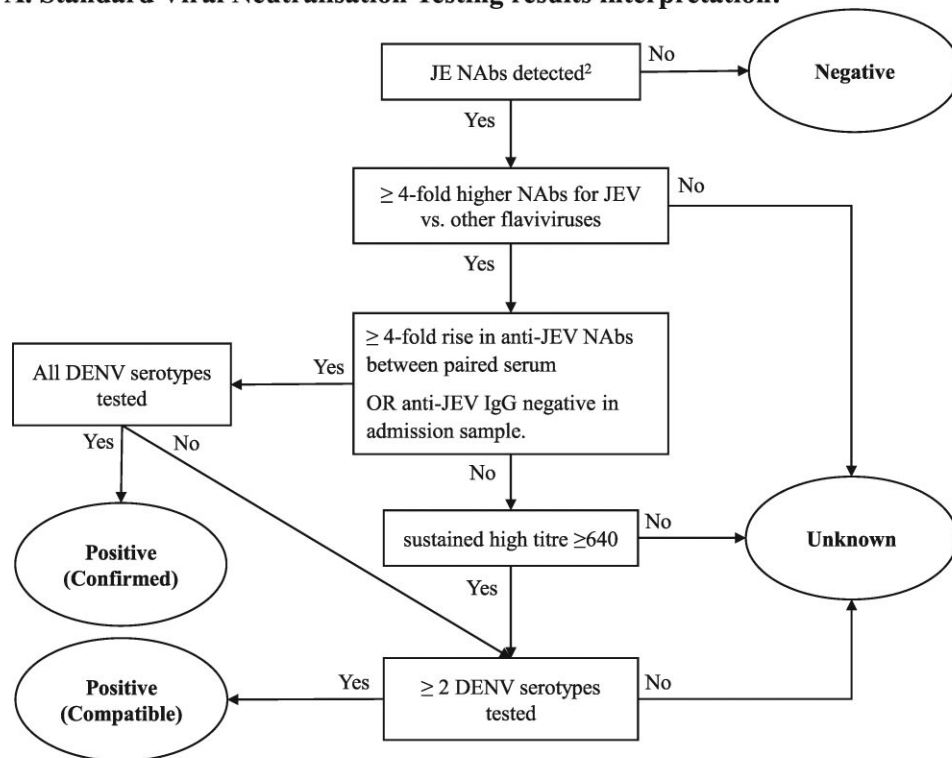
For the subset of 32 patients classified as JE positive, confirmed or compatible (before or after depletion), the median duration of onset of illness was 5 d (IQR 4–7) and the median duration between paired serum samples was 14 d (IQR 11–24). A total of 17/24 (71%) of these patients had detectable anti-JEV IgG in the admission serum before IgG depletion and 23/24 [96%] had detectable anti-JEV IgG in the follow-up sample.

Negative control sera

IgM-VNT was performed on three other groups of negative control sera to assess the specificity of the novel method. JEV NAb was not detected by IgM-VNT or VNT in the healthy flavivirus-naïve blood donors ($n=10$) or ZIKV infection sera ($n=4$) (see Table S3). In the DENV patient sera, 2/12 (17%) did not have detectable JEV NAb, and for both of these patients, IgM-VNT was performed and was also negative. In the 10/12 (83%) patients with DENV infection with JEV NAb detected by VNT, 8/10 (80%) did not have detectable JEV NAb after IgG depletion. For the remaining two, one did not have a result for IgM-VNT and the other showed negative JEV VNT for admission serum and a low JEV NAb titre of 40 in follow-up serum. There were not sufficient sample volumes available to perform DENV VNT.

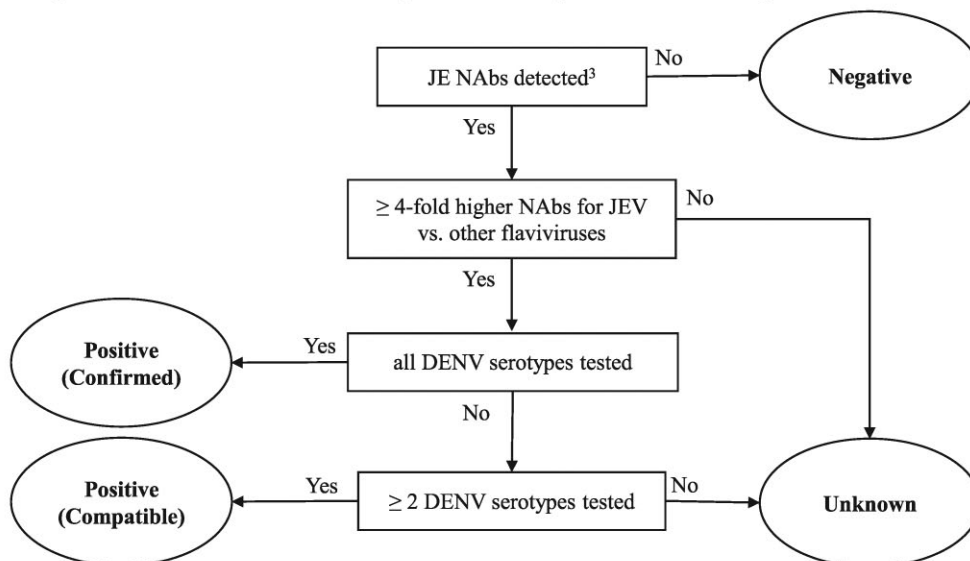
Positive, negative and overall percentage agreement

The IgM-VNT was compared with the reference standard VNT. This was based on results for patients classified as JE positive or negative by standard VNT and with sufficient sera to complete IgM-VNT, i.e. VNT performed after IgG depletion and IgG ELISA to confirm IgG depletion. This included 14 JE-positive and

A. Standard Viral Neutralisation Testing results interpretation:

1. All patients included in the study had anti-JEV IgM detected in CSF or IgM seroconversion from acute to follow-up serum samples, JEV RNA not detected, DENV RNA or NS1 not detected, and no other pathogen detected by extensive molecular and serological testing (42).

2. Nab titer ≥ 40 considered as positive. It would be advised that patients categorised as JE negative by this algorithm should ideally have a serum sample tested at 28 days post symptom onset.

B. IgM Viral Neutralisation Testing results interpretation for a single serum

1. All patients included in the study had anti-JEV IgM detected in CSF or IgM seroconversion from acute to follow-up serum samples, JEV RNA not detected, DENV RNA or NS1 not detected, and no other pathogen detected by extensive molecular and serological testing (42).

2. IgM VNT consists in the performance of VNT on serum samples after IgG depletion confirmed by a negative anti-JEV IgG ELISA result.

3. Nab titer ≥ 40 considered as positive. It would be advised that patients categorised as JE negative by this algorithm should ideally have a serum sample tested at 28 days post symptom onset.

Figure 2. Criteria for interpretation of the results and patient categorisation for JE status.

Table 2. VNT antibody titre in acute and follow-up serum samples for patients with positive anti-JEV IgM capture ELISA

Patient number	Sample type	Days of illness	Class	Before IgG depletion (standard VNT)										After IgG depletion (IgM-VNT)									
				JEV IgG	NAb titre								Class	JEV IgG	NAb titre								
					JEV	D1	D2	D3	D4	ZIK	WN	JEV			D1	D2	D3	D4	ZIK	WN			
1597	Adm	5	Conf	—	1280	Neg	Neg	Neg	Neg			Conf	— ^a	160 ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b		Neg ^b		
1704	FU	59		+	<u>2560</u>	14	14	Neg	20					640 ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b					
	Adm	5	Conf	+	640	20	Neg	20	28			Conf	—	640 ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b		
829	FU	13		+	<u>2560</u>	40	Neg	28	80					<u>2560^b</u>	20 ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b		
	Adm	4	Conf	—	1280	Neg	Neg	Neg	Neg	Neg	14	Conf	—	640 ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b		
908	FU	21		+	<u>2560</u>	Neg	Neg	Neg	14	Neg ^b													
	Adm	4	Conf	+	40	160	160	40	113	Neg		Conf											
928	FU	14		+	<u>2560</u>	20	Neg	Neg	14				—	2560	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b		
	Adm	5	Conf	—	1810	20	Neg	Neg	Neg	Neg	56	Conf	—	<u>2560^b</u>	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b		
2078	FU	44		+	<u>2560</u>	Neg	Neg	Neg	14				—	<u>2560</u>	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b		
	Adm	7	Conf	Eq	160	20	Neg	Neg	Neg			Conf	—										
101	FU	17		+	<u>2560</u>	Neg	Neg	20	20				—	≥2560	Neg	Neg	Neg	Neg					
	Adm	4	Conf	—	2560	Neg	Neg	Neg	Neg			Conf	—	453	Neg	Neg	Neg	Neg					
	FU	6		—	<u>2560</u>	Neg	Neg	40	Neg				—	≥2560	Neg	Neg	Neg	Neg					
1610	Adm	6	Comp	+	<u>2560</u>	14	Neg	Neg	20			Conf	—	1280 ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b		
	FU	40		+	<u>2560</u>	160	20	Neg	113					2560	Neg	Neg	Neg	Neg	Neg	Neg	Neg		
483	Adm	7	Comp	+	<u>2560</u>	Neg	Neg	Neg	Neg			Conf	—	<u>2560^b</u>	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b		
	FU	21		+	<u>2560</u>	20	20	Neg	20	Neg	Neg			<u>2560^b</u>	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b		
884	Adm	5	Comp	+	<u>2560</u>	40	Neg	Neg	40	Neg	40	Conf	—	1280 ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b		
	FU	19		+	<u>2560</u>	Neg	Neg	Neg	20				—	<u>2560^b</u>	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b		
1074	Adm	6	Comp	Eq	452		Neg ^b	Neg	Neg			Conf											
	FU	84		+	1810	40	40	Neg	80				—	1280 ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b		Neg ^b			
1180	Adm	7	Comp	+	1280	452	640	452	160	Neg		Conf	—	1280 ^b	Neg ^b	Neg ^b	Neg ^b	Neg ^b		Neg ^b			
	FU	13		+	<u>2560</u>	226	226	160	160	20	80												
2053	Adm	5	Comp	+	<u>2560</u>	20	Neg	Neg	Neg			Conf	—	<u>2560</u>	Neg	Neg	Neg	Neg					
	FU	18		+	1280	80	Neg	40	Neg				—										
775	Adm	3	Comp	—	320	Neg	Neg		Neg			Comp	— ^a	113	Neg	Neg	Neg						
	FU	12		+	<u>640</u>	Neg	Neg		40					<u>640</u>	Neg	Neg	Neg						
5149	Adm	1	Unkn	+	<u>2560</u>	<u>2560</u>	<u>2560</u>	<u>2560</u>	<u>2560</u>			Conf		1280 ^b	20 ^b	20 ^b	20 ^b	Neg ^b					
	FU	28		+	<u>2560</u>	<u>2560</u>	<u>2560</u>	<u>2560</u>	<u>2560</u>				—	<u>2560^b</u>	40 ^b	20 ^b	40 ^b	20 ^b		Neg ^b			
1056	Adm	3	Unkn	+	320	1810	320	320	320	28	40	Conf	—	640 ^b	20 ^b	Neg ^b	Neg ^b	Neg ^b		Neg ^b			
	FU	28		+	<u>2560</u>																		
1917	Adm	7	Unkn	+	<u>2560</u>	<u>2560</u>	<u>2560</u>	Neg	453			Conf	—	<u>2560</u>	Neg	Neg	Neg	Neg					
	FU	12		+	1280	<u>2560</u>	<u>2560</u>	Neg	320				—	1280	Neg	Neg	Neg	Neg					
1036	Adm	4	Unkn	+	80	<u>2560</u>	226	320	226	Neg	20	Neg	—	Neg ^b	80 ^b	Neg ^b	20 ^b	Neg ^b		Neg ^b			
	FU	16		+	640	<u>2560^b</u>	1280	<u>2560</u>															

Adm: serum on admission; FU: serum at follow-up; NAb: NAb assessed by VNT, geometric mean calculated from duplicate results, = indeterminate, NAb titre underlined to indicate the maximum dilution tested, neg: no NAb detected in duplicate samples (observation of cytopathic effect) for all serum dilutions tested (lowest = 20); NAb titre ≥40 considered as positive; D1–4: dengue virus 1–4; ZIK: Zika virus; WN: West Nile virus; class: classification for JE status according to criteria in Table 2; Conf: confirmed; Comp: compatible; Unkn: unknown; JEV IgG: anti-JEV IgG detection by ELISA (Euroimmun); +: positive; Eq: equivocal; —: negative.

^a JEV IgG negative before depletion.

^b Only one replicate tested or interpretable, the other samples were tested in duplicate.

16 JE-negative patients. Positive, negative and overall percentage agreements (PPA, NPA and OPA, respectively) were all 100% (see Table 3).

VNT after IgG depletion for patients with single acute serum

A total of 76/98 (78%) patient samples had sufficient volumes for IgG depletion, confirmatory IgG ELISA testing and IgM-VNT.

Results allowed classification for 72/76 (95%) patients: 70 (92%) JE, 63 (83%) confirmed and 3 (4%) compatible, and 6 (8%) negative. Four (5%) were unknown (Table 4).

Discussion

This pilot study included a large set of well-characterized patients recruited prospectively in clinical studies, with extensive VNT for

Table 3. 2×2 table of the results of IgM-VNT as compared with standard VNT

IgM-VNT	Reference test (standard VNT)		Total, n
	JE positive ^a , n	JE negative ^a , n	
JE positive ^a	14	0	14
JE negative ^a	0	16	16
Total	14	16	30

^aThe classification of patients followed the criteria set out in Figure 2.

JEV, DENV 1–4, ZIKV and WNV. We show that the implementation of IgG depletion prior to VNT performed on par with standard VNT (100% PPA, NPA and OPA) and also resulted in a significantly higher proportion, compared with standard VNT, of patients being classified. Of the patients with paired sera tested to confirm acute JEV infection, 74% (26/35) were classified without an IgG depletion step, in contrast to 100% when IgG depletion was included. Furthermore, IgG depletion improved the diagnostic confidence of patients classed as JE positive, from 7/26 (27%) confirmed as opposed to 19/26 (73%) compatible with standard VNT to 16/17 (94%) confirmed as opposed to 1/17 (6%) compatible with IgM-VNT. Depleting IgG also enabled a diagnosis of JE in 95% of patients for whom only a single sample was available, allowing for specific neutralization of the IgM remaining in the sample.

The high proportion of patients presenting with detectable anti-JEV IgG before depletion and a reduction in DENV neutralization titres after depletion strengthen the underlying premise of this study, that IgG complicates discrimination by VNT, especially in areas with high endemicity of other flaviviruses and increasing utilization of JEV vaccination.

A limitation is that there were not sufficient sample volumes available to perform standard and IgM-VNT in all samples. However, the testing was retrospectively performed on a relatively large number of very precious samples. It would be realistic in clinical practice to secure the serum volume (400 µL) needed for prospective IgM-VNT testing. This is one of the advantages of the new technique, that it relies on a single serum sample rather than paired sera or CSF. The efficiency of the IgG depletion was evaluated using anti-JEV IgG ELISA. We found that 84% of the anti-JEV IgG ELISA-positive sera became negative after IgG depletion. All samples with an anti-JEV IgG ELISA result <125 RU/mL were negative after IgG depletion, suggesting IgG depletion was probably incomplete in samples with high titres. Further optimization is required to ensure that depletion is fully effective, perhaps with alternative methods depending on the initial anti-JEV IgG result, such as the use of three rather than two IgG depletion columns.

The principle of removing IgG and the use of IgM as a biomarker for confirming acute infection is by no means novel. In 1973, Edelman and Pariyanonda⁴⁹ reported a modified haemagglutination inhibition involving depletion of IgG by sucrose density

gradient centrifugation of whole serum and 2-mercaptoethanol treatment. The improved discrimination of evidence for acute JE in patient samples gave rise to further work developing the widely used anti-JEV IgM ELISA.^{50,51} However, with evidence suggesting suboptimal performance of MAC-ELISA,¹² the increasing use of the JEV vaccine, as well as hyperendemicity of DENV serotypes, the requirement for accurate diagnostic confirmation becomes even more pertinent. Although the performance of contemporaneous anti-DENV IgM ELISA and calculation of a JEV:DENV IgM ratio has improved specificity, the combination of VNT and IgG depletion (IgM-VNT) permits IgM detection with higher specificity than by using MAC-ELISA alone.

Calvert et al.¹⁹ showed that IgG depletion prior to neutralization testing considerably improved (15% before to 77% after IgG depletion) the differentiation of acute Zika from dengue viral infections. This has also been demonstrated for DENV infections.^{42,43} It is notable that as JE is predominantly a neurological infection, and the natural history of the immunological response is different to flavivirus infections presenting as acute febrile syndromes, by the time of clinical presentation, anti-JEV IgM and IgG is detectable in a larger proportion of patients. Therefore use of the IgM-VNT method for JE confirmation is a logical approach.

The humoral responses to JEV infection are directed mainly against antigenic epitopes on the viral envelope protein. There is major cross-reactivity with other endemic circulating flaviviruses and therefore it was crucial to test for all DENV serotypes,^{52,53} ZIKV⁵⁴ and WNV⁴⁴ where they are sympatric. Likewise, IgG depletion and seroneutralization might play a role in the diagnosis of DENV neurological infections for which there is considerable diagnostic uncertainty.

We acknowledge that a diagnostic accuracy study should ideally be performed with an a priori sample size calculation, prospectively testing consecutive patients with suspected neurological infection by the reference standard VNT to ascertain JE-positive and negative patient samples. However, we were unable to conduct this in this pilot study and flavivirus-naïve patients from France were included as an additional category of negative controls. That patients already had anti-JEV IgM detected in CSF or experienced JEV seroconversion reflects the role of VNT within reference centres. Further limitations include missing data due to limited sample volumes and that dilutions were 1/20 to 1/2560 for the sera. Ideally serum should be tested to the end point of dilution. IgM-VNT is a diagnostic test suited for reference centres and optimization will be required to adapt the technique to be high throughput, using protein G slurry and an automatized format for VNT testing of 1/20 to 1/5120. Additionally, not all the virus strains used were sourced from the countries where the samples were derived; the DENV strains isolated from Laos did not provide a sufficient cytopathic effect for the assay and neither ZIKV nor WNV have been isolated from patients in Laos.

In conclusion, measurement of anti-JEV IgG and the performance of IgM-VNT significantly improved performance and allowed the use of a single serum sample instead of paired sera for JE confirmation. This innovation holds promise for wider incorporation into testing algorithms in the reference confirmation of JE and DENV neurological infections.

Table 4. VNT antibody titre for patients with only a single acute serum sample

Patient number	Days of illness	Class	Before IgG depletion, JEV IgG	After IgG depletion (IgM-VNT)							
				JEV IgG	NAb titre						
					JEV	D1	D2	D3	D4	ZIK	WN
34		Conf	—	—	160	Neg	Neg	Neg	Neg	Neg	Neg
37	3	Conf	—	—	640	Neg	Neg	Neg	Neg	Neg	Neg
38	2	Conf	—	—	57	Neg	Neg	Neg	Neg	Neg	Neg
40	14	Conf	—	—	80	Neg	Neg	Neg	Neg	Neg	Neg ^a
44	4	Conf	—	—	57	Neg	Neg	Neg	Neg	Neg	Neg
47	4	Conf	—	—	320	Neg	Neg	Neg	Neg	Neg	Neg
52	4	Conf	—	—	80	Neg	Neg	Neg	Neg	Neg	Neg
53	4	Conf	—	—	57	Neg	Neg	Neg	Neg	Neg	Neg
59	4	Conf	—	—	320	Neg	Neg	Neg	Neg	Neg	Neg
60	1	Conf	—	—	160	Neg	Neg	Neg	Neg	Neg	Neg
64	3	Conf	—	—	80	Neg	Neg	Neg	Neg	Neg	Neg
57	6	Conf	—	—	640	Neg	Neg	Neg	Neg	Neg	Neg
66	8	Conf	—	—	40	Neg	Neg	Neg	Neg	Neg	Neg
73	5	Conf	—	—	1810	Neg	Neg	Neg	Neg	Neg	Neg
76	5	Conf	—	—	320	Neg	Neg	Neg	Neg	Neg	Neg
87	4	Conf	—	—	57	Neg	Neg	Neg	Neg	Neg	Neg
88	6	Conf	—	—	160	Neg	Neg	Neg	Neg	Neg	Neg
92	3	Conf	—	—	905	Neg	Neg	Neg	Neg	Neg	Neg
98		Conf	—	—	320	Neg	Neg	14	Neg	Neg	Neg
101		Conf	—	—	1280	Neg	Neg	Neg	Neg	Neg	Neg
102		Conf	—	—	640	Neg	Neg	28	Neg	Neg	Neg
103		Conf	—	—	320	Neg	Neg	Neg	Neg	Neg	Neg
104		Conf	—	—	226	Neg	Neg	Neg	Neg	Neg	Neg
105		Conf	—	—	160	Neg	Neg	Neg	14	Neg	Neg
111		Conf	—	—	452	Neg	Neg	Neg	Neg	Neg	Neg
112		Conf	—	—	160	Neg	Neg	Neg	Neg	Neg	Neg
127		Conf	—	—	640	Neg	Neg	Neg	Neg	Neg	14
118		Conf	—	—	640	Neg	Neg	Neg	14	Neg	Neg
89	3	Conf	—	— ^b	160	Neg	Neg	Neg	Neg	Neg	Neg
97		Conf	—	— ^b	640	Neg	Neg	Neg	Neg	Neg	Neg
94	3	Conf	—	— ^b	640	Neg	Neg	Neg	Neg	Neg	Neg
110		Conf	—	— ^b	160	Neg	Neg	Neg	Neg	Neg	Neg
121		Conf	—	— ^b	160	14	Neg	Neg	Neg		
54	3	Conf		—	57	Neg	Neg	Neg	Neg	Neg	Neg
128		Conf	Eq	—	640	Neg	Neg	Neg	Neg	Neg	Neg
51	5	Conf	Eq	—	905	Neg	Neg	Neg	Neg	Neg	Neg
33		Conf	Eq	—	452	Neg	Neg	Neg	Neg	Neg	Neg
58	5	Conf	Eq	—	2560 ^c	Neg	Neg	Neg	Neg	Neg	Neg
62	6	Conf	Eq	—	226	20	Neg	Neg	Neg	Neg	Neg
35	4	Conf	+	—	160	Neg	Neg	Neg	Neg	Neg	Neg
36		Conf	+	—	40	Neg	Neg	Neg	Neg	Neg	Neg
41	4	Conf	+	—	320	Neg	Neg	Neg	Neg	Neg	Neg
65	13	Conf	+	—	80	14	Neg	14	Neg	Neg	Neg
67	4	Conf	+	—	1280	Neg	Neg	Neg	Neg	Neg	28
68	6	Conf	+	—	2560 ^c	Neg	Neg	Neg	Neg	Neg	Neg
69	5	Conf	+	—	452	Neg	Neg	Neg	Neg	Neg	Neg
70	6	Conf	+	—	640	Neg	Neg	Neg	Neg	Neg	Neg
71	8	Conf	+	—	640	Neg	Neg	Neg	Neg	Neg	14
74	4	Conf	+	—	226	Neg	Neg	Neg	Neg	Neg	Neg

Table 4. Continued

Patient number	Days of illness	Class	Before IgG depletion, JEV IgG	After IgG depletion (IgM-VNT)							
				JEV IgG	NAb titre						
					JEV	D1	D2	D3	D4	ZIK	WN
75	14	Conf	—	—	905	Neg	Neg	Neg	Neg	Neg	Neg
79	5	Conf	+	—	640	Neg	Neg	Neg	Neg	Neg	Neg
80	7	Conf	+	—	226	Neg	Neg	Neg	Neg	Neg	Neg
81	6	Conf	+	—	640	Neg	Neg	Neg	Neg	Neg	Neg
82	6	Conf	+	—	905	Neg	Neg	Neg	Neg	Neg	Neg
86		Conf	+	—	2560 ^c	Neg	Neg	Neg	Neg	Neg	20
91	7	Conf	+	—	1280	Neg	Neg	Neg	Neg	Neg	Neg
95	4	Conf	+	—	320	Neg	Neg	Neg	Neg	Neg	14
99		Conf	+	—	640	40	Neg	Neg	20	Neg	Neg
108		Conf	+	—	320	Neg	Neg	Neg	40	Neg	Neg
109		Conf	+	—	113	Neg	Neg	Neg	Neg	Neg	Neg
116		Conf	+	—	113	Neg	Neg	Neg	Neg	Neg	Neg
122		Conf	+	—	1280	Neg	Neg	Neg	Neg	Neg	Neg
125		Conf	+	—	160	Neg	Neg	14	Neg	Neg	Neg
31		Comp	—	—	226		Neg	Neg	Neg ^d		
32		Comp	—	—	226		Neg	Neg	Neg ^d		
45	10	Comp	—	—	80		Neg	Neg	Neg		
56	6	Unkn	+	—	80	Neg	40	20	Neg	Neg	Neg
77	6	Unkn	+	—	80	Neg	28	Neg	Neg	Neg	Neg
85		Unkn	+	—	320	98	160	57	20	Neg	Neg
78	4	Unkn	+	—	160	Neg	Neg	Neg	Neg	80	Neg
39	3	Neg	—	—	Neg	Neg	Neg	Neg	Neg	Neg	Neg
83	5	Neg	—	—	Neg	Neg	Neg	Neg	Neg	Neg	Neg
48	3	Neg	+	—	Neg	20	Neg	Neg	Neg	Neg	Neg
61	14	Neg	+	—	Neg	14	14	Neg	14	Neg	Neg
49	10	Neg	+	—	Neg	14	Neg	Neg	Neg		
120		Neg	+	—	20	40	Neg	20	14	Neg	Neg

NAb titre: NAb assessed by VNT, geometric mean calculated from duplicate results. neg: no NAb detected in duplicate samples (observation of cytopathic effect) for all serum dilutions tested (lowest = 20); NAb titre ≥ 40 considered as positive; D1–4: dengue virus 1–4; ZIK: Zika virus; WN: West Nile virus; class: classification for JE status according to the criteria set out in Figure 2; Conf: confirmed; Comp: compatible; Unkn: unknown; JEV IgG: anti-JEV IgG detection by ELISA (Euroimmun); +: positive; Eq: equivocal; —: negative.

^aOnly one replicate tested or interpretable, the other samples were tested in duplicate.

^bJEV IgG negative before depletion.

^cMaximum dilution tested.

^dTest not performed.

Supplementary data

Supplementary data are available at [Transactions](https://www.transactions.org) online.

provided the clinical samples. TB, NA and BP performed the experimental work. TB, NA, ADP, BP, NZ and XDL analysed and interpreted the data. TB wrote the manuscript. All the authors edited successive drafts and approved the final version.

Authors' contributions: TB, ADP and XDL conceived the study. TB, NA, ADP, BP, XDL and NZ developed the methodology. SR, MV, MM, AC, OS, OP, ADP, JDP, CG and PNN designed and conducted the clinical study and

Acknowledgements: We are very grateful to the patients and to Bounthaphany Bounxouei, the former Director of Mahosot Hospital, the late Rattanaphone Phetsouvanh, Director of the Microbiology Laboratory, and the staff of the wards and Microbiology Laboratory of Mahosot Hospi-

tal. We also thank Bounnak Saysanasongkham, the former Director of the Department of Healthcare and Rehabilitation, Ministry of Health, and Bounkong Syhavong, Minister of Health, Lao PDR for their very kind help and support. We thank the stakeholders of the SEAE project,⁴⁵ members of the Unité des Virus Émergents (Christine Isnard and Camille Placidi) and the CNR des Arbovirus (Patrick Gravier, Gilda Grard, Isabelle Leparc-Goffart and Mathilde Galla). We also thank Rodrigo Cachay, Eduardo Gottuzo and Humberto Guerra (Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia) for providing the Zika virus patient samples.

Collaborators: We are grateful to all the SEAE study researchers, including Philippe Buchy, Em Bunnakea, Julien Cappelle, Mey Channa, Veronique Chevalier, Yoann Crabol, Philippe Dussart, Marc Eloit, Magali Herrant, Nguyen Hien, Chaw Su Hlaing, Jérôme Honnorat, Tran Thi Mai Hung, Tran Thi Thu Huong, Latt Latt Kyaw, Nguyen Van Lam, Denis Laurent, Marc Lecuit, Kyaw Linn, Olivier Lortholary, Aye Mya Min Aye, Philippe Perot, Sommanikhone Phangmanixay, Khounthavy Phongsavath, Phan Huu Phuc, Anne-Laurie Pinto, Patrice Piola, Bruno Rosset, Ky Santy, Heng Sothy, Arnaud Tarantola, Nguyen Thi Thu Thuy, Htay Htay Tin, Ommar Swe Tin, Pham Nhat An, Dang Duc Anh, Pascal Bonnet, Kimrong Bun, Danoy Chommanam, Viengmon Davong, Patrice Debré, Jean-François Delfraissy, Christian Devaux, Anousone Douangnouvong, Veasna Duong, Benoit Durand, Chanreasmey Eng, Catherine Ferrant, Didier Fontenille, Lukas Hafner, Le Thanh Hai, Do Thu Huong, Marc Jouan, May July, Magali Lago, Jean-Paul Moatti, Bernadette Murgue, Khin Yi Oo, MengHeng Oum, Khansoudaphone Phakhounthong, Anh Tuan Pham, Do Quyen, Malee Seephonelee, Maud Seguy, Bountoy Sibounheunang, Kanarith Sim, Luong Minh Tan, Cho Thair, Win Thein, Phung Bich Thuy, Hervé Tissot-Dupont and Malavanh Vongsouvath.

Funding: The work was supported by the University of Oxford and the Medical Research Council (grant MR/N013468/1). It was also supported by the Oxford Glycobiology endowment, the Institute of Research for Development, Aix-Marseille University, the Wellcome Trust of Great Britain and the European Union's Horizon 2020 research, Fondation Total, Institut Pasteur, International Network Institut Pasteur, Fondation Merieux, Aviesan Sud, Institut national de la santé et de la recherche médicale (Inserm), and innovation programme EVAg (grant agreement 653316). The Zika virus patient samples were provided by the EC-funded project ZIKAlliance, Grant agreement no. 734548.

Competing interests: None declared.

Ethical approval: Ethical clearance for the Laos CNS study was granted by the Ethical Review Committee of the former Faculty of Medical Sciences, National University of Laos (now University of Health Sciences) and the Oxford University Tropical Ethics Research Committee, Oxford, UK. For the blood donor samples, the protocol was presented to an ethical committee (Comité de Protection des Personnes Sud Méditerranée I) and because no additional blood sampling was required, the committee agreed that ethical approval was not required. The protocol is in agreement with the national regulations on personal data (Commission Nationale Informatique et Liberté), the collection of biological samples was declared to the French Ministry of Research and all data and samples were anonymized. For the Zika sera, ethical approval was granted by the Institutional Ethics Committee of the Universidad Peruana Cayetano Heredia (SIDISI 103488).

Data availability: The data underlying this article are available in the article and in its online supplementary material.

References

- World Health Organization. Japanese encephalitis vaccines: WHO position paper, February 2015–recommendations. *Vaccine*. 2016;34(3):302–3.
- Heffelfinger JD, Li X, Batmunkh N, et al. Japanese encephalitis surveillance and immunization – Asia and Western Pacific regions, 2016. *MMWR Morb Mortal Wkly Rep*. 2017;66(22):579–83.
- Hills SL, Walter EB, Atmar RL, et al. Japanese encephalitis vaccine: recommendations of the Advisory Committee on Immunization Practices. *MMWR Recomm Rep*. 2019;68(2):1–33.
- Pearce JC, Learoyd TP, Langendorf BJ, et al. Japanese encephalitis: the vectors, ecology and potential for expansion. *J Travel Med*. 2018;25(Suppl 1):S16–26.
- Simon-Loriere E, Faye O, Prot M, et al. Autochthonous Japanese encephalitis with yellow fever coinfection in Africa. *N Engl J Med*. 2017;376(15):1483–5.
- Kulkarni R, Sapkal GN, Kaushal H, et al. Japanese encephalitis: a brief review on Indian perspectives. *Open Virol J*. 2018;12(1):121–30.
- Fang Y, Zhang Y, Zhou ZB, et al. New strains of Japanese encephalitis virus circulating in Shanghai, China after a ten-year hiatus in local mosquito surveillance. *Parasites Vectors*. 2019;12:14.
- Ojha JK, Samantaray K, Mohanty S. Assess the coverage rate of Japanese encephalitis vaccination and factors of non-compliance as reported by parents of selected areas of Khurda. *Eur J Mol Clin Med*. 2021;7(11):5049–60.
- Do LP, Bui TM, Hasebe F, et al. Molecular epidemiology of Japanese encephalitis in northern Vietnam, 1964–2011: genotype replacement. *Virol J*. 2015;12:51.
- Bharucha T, Sengvilaipaseuth O, Vongsouvath M, et al. Development of an improved RT-qPCR Assay for detection of Japanese encephalitis virus (JEV) RNA including a systematic review and comprehensive comparison with published methods. *PLoS One*. 2018;13(3):e0194412.
- Hills S, Dabbagh A, Jacobson J, et al. Evidence and rationale for the World Health Organization recommended standards for Japanese encephalitis surveillance. *BMC Infect Dis*. 2009;9:214.
- Dubot-Peres A, Sengvilaipaseuth O, Chanthongthip A, et al. How many patients with anti-JEV IgM in cerebrospinal fluid really have Japanese encephalitis? *Lancet Infect Dis*. 2015;15(12):1376–7.
- Maeki T, Tajima S, Ikeda M, et al. Analysis of cross-reactivity between flaviviruses with sera of patients with Japanese encephalitis showed the importance of neutralization tests for the diagnosis of Japanese encephalitis. *J Infect Chemother*. 2019;25(10):786–90.
- Hills S, Van Keulen A, Feser J, et al. Persistence of IgM antibodies after vaccination with live attenuated Japanese encephalitis vaccine. *Am J Trop Med Hyg*. 2020;104(2):576–9.
- Fatima T, Rais A, Khan E, et al. Investigation of Japanese encephalitis virus as a cause of acute encephalitis in southern Pakistan, April 2015–January 2018. *PLoS One*. 2020;15(6):e0234584.
- Centers for Disease Control. Japanese encephalitis. Diagnostic testing. Available from: <https://www.cdc.gov/japaneseencephalitis/healthcareproviders/healthcareproviders-diagnostic.html> [accessed 18 April 2022].
- Robinson JS, Featherstone D, Vasanthapuram R, et al. Evaluation of three commercially available Japanese encephalitis virus IgM enzyme-linked immunosorbent assays. *Am J Trop Med Hyg*. 2010;83(5):1146–55.

- 18 Bharucha T, Shearer FM, Vongsouvath M, et al. A need to raise the bar—a systematic review of temporal trends in diagnostics for Japanese encephalitis virus infection, and perspectives for future research. *Int J Infect Dis.* 2020;95:444–56.
- 19 Calvert AE, Boroughs KL, Laven J, et al. Incorporation of IgG depletion in a neutralization assay facilitates differential diagnosis of Zika and dengue in secondary flavivirus infection cases. *J Clin Microbiol.* 2018;56(6):e00234–18.
- 20 Balakrishnan A, Thekkekkare RJ, Sapkal G, et al. Seroprevalence of Japanese encephalitis virus & West Nile virus in Alappuzha district, Kerala. *Indian J Med Res.* 2017;146(Suppl):S70–5.
- 21 Meyding-Lamadé U, Craemer E, Schnitzler P. Emerging and re-emerging viruses affecting the nervous system. *Neurol Res Pract.* 2019;1:20.
- 22 Amaro F, Sanchez-Seco MP, Vazquez A, et al. The application and interpretation of IgG avidity and IgA ELISA tests to characterize Zika virus infections. *Viruses.* 2019;11(2):179.
- 23 Warnecke JM, Lattwein E, Saschenbrecker S, et al. Added value of IgA antibodies against Zika virus non-structural protein 1 in the diagnosis of acute Zika virus infections. *J Virol Methods.* 2019;267:8–15.
- 24 Colonetti T, Rocha BVE, Grande AJ, et al. Accuracy of immunoglobulin M and immunoglobulin A of saliva in early diagnosis of dengue: systematic review and meta-analysis. *An Acad Bras Cienc.* 2018;90(3):3147–54.
- 25 Nascimento EJM, Huleatt JW, Cordeiro MT, et al. Development of antibody biomarkers of long term and recent dengue virus infections. *J Virol Methods.* 2018;257:62–8.
- 26 Rockstroh A, Moges B, Barzon L, et al. Specific detection of dengue and Zika virus antibodies using envelope proteins with mutations in the conserved fusion loop. *Emerg Microbes Infect.* 2017;6(11):e99.
- 27 Zhang B, Pinsky BA, Ananta JS, et al. Diagnosis of Zika virus infection on a nanotechnology platform. *Nat Med.* 2017;23(5):548–50.
- 28 Huang CH, Chang YH, Lin CY, et al. Shared IgG infection signatures vs. hemorrhage-restricted IgA clusters in human dengue: a phenotype of differential class-switch via *TGFβ1*. *Front Immunol.* 2017;8:1726.
- 29 Balmaseda A, Saborio S, Tellez Y, et al. Evaluation of immunological markers in serum, filter-paper blood spots, and saliva for dengue diagnosis and epidemiological studies. *J Clin Virol.* 2008;43(3):287–91.
- 30 Balmaseda A, Guzman MG, Hammond S, et al. Diagnosis of dengue virus infection by detection of specific immunoglobulin M (IgM) and IgA antibodies in serum and saliva. *Clin Diagn Lab Immunol.* 2003;10(2):317–22.
- 31 Yap G, Sil BK, Ng LC. Use of saliva for early dengue diagnosis. *PLoS Negl Trop Dis.* 2011;5(5):e1046.
- 32 de Vasconcelos ZFM, Azevedo RC, Thompson N, et al. Challenges for molecular and serological ZIKV infection confirmation. *Childs Nerv Syst.* 2018;34(1):79–84.
- 33 Ronnberg B, Gustafsson A, Vapalahti O, et al. Compensating for cross-reactions using avidity and computation in a suspension multiplex immunoassay for serotyping of Zika versus other flavivirus infections. *Med Microbiol Immunol.* 2017;206(5):383–401.
- 34 Tsai WY, Youn HH, Tyson J, et al. Use of urea wash ELISA to distinguish Zika and dengue virus infections. *Emerg Infect Dis.* 2018;24(7):1355–9.
- 35 Shen WF, Galula JU, Chang GJ, et al. Improving dengue viral antigens detection in dengue patient serum specimens using a low pH glycine buffer treatment. *J Microbiol Immunol Infect.* 2017;50(2):167–74.
- 36 Balmaseda A, Stettler K, Medialdea-Carrera R, et al. Antibody-based assay discriminates Zika virus infection from other flaviviruses. *Proc Natl Acad Sci USA.* 2017;114(31):8384–9.
- 37 Zhu T, He J, Chen W, et al. Development of peptide-based chemiluminescence enzyme immunoassay (CLEIA) for diagnosis of dengue virus infection in human. *Anal Biochem.* 2018;556:112–8.
- 38 Lebani K, Jones ML, Watterson D, et al. Isolation of serotype-specific antibodies against dengue virus non-structural protein 1 using phage display and application in a multiplexed serotyping assay. *PLoS One.* 2017;12(7):e0180669.
- 39 Piyasena TBH, Setoh YX, Hobson-Peters J, et al. Differential diagnosis of flavivirus infections in horses using viral envelope protein domain III antigens in enzyme-linked immunosorbent assay. *Vector Borne Zoonotic Dis.* 2017;17(12):825–35.
- 40 Kim DTH, Bao DT, Park H, et al. Development of a novel peptide aptamer-based immunoassay to detect Zika virus in serum and urine. *Theranostics.* 2018;8(13):3629–42.
- 41 Fietze KM, Pascale JM, Moreno B, et al. Pathogen-specific deep sequence-coupled biopanning: a method for surveying human antibody responses. *PLoS One.* 2017;12(2):e0171511.
- 42 Tsai W-Y, Durbin A, Tsai J-J, et al. Complexity of neutralizing antibodies against multiple dengue virus serotypes after heterotypic immunization and secondary infection revealed by in-depth analysis of cross-reactive antibodies. *J Virol.* 2015;89(14):7348–62.
- 43 Zainal N, Tan KK, Johari J, et al. Sera of patients with systemic lupus erythematosus cross-neutralizes dengue viruses. *Microbiol Immunol.* 2018;62(10):659–72.
- 44 Dubot-Peres A, Mayxay M, Phetsouvanh R, et al. Management of central nervous system infections, Vientiane, Laos, 2003–2011. *Emerg Infect Dis.* 2019;25(5):898–910.
- 45 Pommier JD, Gorman C, Crabol J, et al. on behalf of the SEAE Consortium. An extensive three-year investigation of childhood encephalitis in the Greater Mekong region - The South East Asia encephalitis project. *Lancet Global Health* (in press).
- 46 Cachay R, Schwalb A, Acevedo-Rodriguez JG, et al. Zika virus seroprevalence in two districts of Chincha, Ica, Peru: a cross-sectional study. *Am J Trop Med Hyg.* 2021;106(1):192–8.
- 47 Nurtop E, Villarroel PMS, Pastorino B, et al. Combination of ELISA screening and seroneutralisation tests to expedite Zika virus seroprevalence studies. *Virology J.* 2018;15(1):192.
- 48 Sakhria S, Bichaud L, Mensi M, et al. Co-circulation of Toscana virus and Punique virus in northern Tunisia: a microneutralisation-based seroprevalence study. *PLoS Negl Trop Dis.* 2013;7(9):e2429.
- 49 Edelman R, Pariyanonda A. Human immunoglobulin M antibody in the sero-diagnosis of Japanese encephalitis virus infections. *Am J Epidemiol.* 1973;98(1):29–38.
- 50 Burke DS, Nisalak A, Ussery MA. Antibody capture immunoassay detection of Japanese encephalitis virus immunoglobulin M and G antibodies in cerebrospinal fluid. *J Clin Microbiol.* 1982;16(6):1034–42.
- 51 Burke DS, Nisalak A. Detection of Japanese encephalitis virus immunoglobulin M antibodies in serum by antibody capture radioimmunoassay. *J Clin Microbiol.* 1982;15(3):353–61.
- 52 Castonguay-Vanier J, Klitting R, Sengvilaipaseuth O, et al. Molecular epidemiology of dengue viruses in three provinces of Lao PDR, 2006–2010. *PLoS Negl Trop Dis.* 2018;12(1):e0006203.
- 53 Mayxay M, Castonguay-Vanier J, Chansamouth V, et al. Causes of non-malarial fever in Laos: a prospective study. *Lancet Glob Health.* 2013;1(1):e46–54.
- 54 Pastorino B, Sengvilaipaseuth O, Chanthongthip A, et al. Low Zika virus seroprevalence in Vientiane, Laos, 2003–2015. *Am J Trop Med Hyg.* 2019;100(3):639–42.