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

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Background: The mainstay of diagnostic confirmation of acute Japanese encephalitis (JEV) involves detection of anti-JE 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 (JEV n=35) and negative controls of healthy flavivirus-naive (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. Nonetheless, gaps remain in understanding the epidemiology of the disease. Incorporation of JEV immunization in routine schedules and coverage remain suboptimal and there is inadequate surveillance to identify vaccine failure and JEV geographical expansion. Detection of JEV nucleic acid is highly specific and provides additional molecular information. However, viraemia is brief and low in humans and JEV RNA is rarely detected. 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. 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. For this reason, the
**Patient selection**

| Laos CNS patients, n=2,364 |
|-----------------------------|
| All patients recruited as part of a prospective study of patients admitted with suspected central nervous system (CNS) infection at Hospitals in Vientiane, Laos, 2003 to 2017. |

| JEV patients, n=264 |
|---------------------|
| Patients positive for anti-JEV IgM in CSF or with seroconversion and negative for other screened etiologies. |

| Included patients, n=133 |
|--------------------------|
| Patients with sufficient serum samples available for viral seroneutralisation testing (VNT) |
| - 35 patients with paired (admission and follow-up) serum samples |
| - 98 with a single admission serum sample |

**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

- 72/125 (58%) admission serum
- 30/31 (97%) of follow-up serum

| Patients with paired sera, n=35 |
|--------------------------------|
| **Standard VNT, n=35** |
| VNT before IgG depletion |
| - 7 (20%) JE confirmed |
| - 18 (51%) JE compatible |
| - 10 (29%) Unknown |

| IgM VNT, n=18* |
|----------------|
| VNT after IgG depletion |
| - 17 (89%) JE confirmed |
| - 1 (5%) JE compatible |
| - 1 (5%) Negative |

* Volume was not sufficient for full testing for 17 patients.

| Patients with single serum, n=98 |
|----------------------------------|
| **IgM VNT*, n=76** |
| VNT after IgG depletion |
| - 63 (83%) JE confirmed |
| - 3 (4%) JE compatible |
| - 4 (5%) Unknown |
| - 6 (8%) Negative |

* VNT before IgG depletion was not performed due to insufficient sample volumes.

** 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.\(^1\)

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. 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). All of these viruses can manifest as neurological complications.

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, IgG subclasses, antibody avidity, incorporation of blocking agents and production of specific monoclonal antibodies for identification of specific viral epitopes. A modification of VNT, involving prior depletion of IgG, has been successfully performed for ZIKV and DENV infections. 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. Patients from 2014 to 2017 were included in the Southeast Asia Encephalitis Project. The laboratory also receives samples from patients from other hospitals around Vientiane City (i.e. Friendship, Children’s and Sethathirat 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 study; 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
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.\(^3,^4\) 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 aetiologies\(^4\) (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-naive 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

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**Table 1. Virus strain used in VNTs**

| Virus | Strain | Country of isolation | GenBank number | EVAg number | Titre (TCID₅₀/mL) | Day read |
|-------|--------|----------------------|----------------|-------------|------------------|----------|
| JEV   | Laos 2009 | Laos | KC196115 | 001V-02217 | 2×10⁵ | 5 |
| WNV   | UVE/WNV/2008/US/R94224 | USA | – | 001V-02224 | 2.1×10⁷ | 5 |
| ZIKV  | ZIKV strain H/PF/2013 French Polynesia | French Polynesia | KJ776791 | – | 3.7×10⁶ | 5 |
| DENV-1 | DENV1 2012 | Saint Vincent and the Grenadines | VC16692 | 001V-02335 | 3.1×10⁷ | 7 |
| DENV-2 | UVE/DENV-2/1998/MQ/703 | Martinique | AF208496 | – | 6.7×10⁴ | 5 |
| DENV-3 | UVE/DENV-3/2001/MQ/2023 | Martinique | AHO11666 | – | 4.5×10⁵ | 6 |
| DENV-4 | UVE/DENV-4/1998/ID/814 | Indonesia | – | – | 3×10⁶ | 6 |

EVAg: European Virus Archive – GLOBAL; TCID₅₀: 50% tissue culture infective dose.
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 pathogens detected by extensive molecular and serological testing (42).

2. Nab titre ≥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 pathogens 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 titre ≥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 | NAb titre | Before IgG depletion (standard VNT) | After IgG depletion (IgM-VNT) |
|---------------|-------------|----------------|-----------|------------------------------------|-------------------------------|
|               |             |                | Class     | JEV IgG | JEV | D1 | D2 | D3 | D4 | ZIK | WN | Class | JEV IgG | JEV | D1 | D2 | D3 | D4 | ZIK | WN |
| 1597          | Adm         | 5              | Conf      | –       | 1280 | Neg | Neg | Neg | Neg | Conf | –a | 160 | Neg | Neg | Neg | Neg | Neg | Neg |
| 1704          | FU          | 59             | +         | 2560   | 14   | 14 | Neg | 20  |     | Conf | –     | 640 | Neg | Neg | Neg | Neg | Neg | Neg |
| 829           | Adm         | 5              | Conf      | –       | 1280 | Neg | Neg | Neg | Neg | 14   | Conf | –     | 640 | Neg | Neg | Neg | Neg | Neg | Neg |
| 908           | FU          | 21             | +         | 2560   | Neg | Neg | 14 | Neg |     | Conf | –     | 2560 | Neg | Neg | Neg | Neg | Neg | Neg |
| 928           | Adm         | 5              | Conf      | –       | 1810 | Neg | Neg | Neg | Neg | 56   | Conf | –     | 2560 | Neg | Neg | Neg | Neg | Neg | Neg |
| 2078          | FU          | 44             | +         | 2560   | Neg | Neg | 14 |     |     | Conf | –     | 2560 | Neg | Neg | Neg | Neg | Neg | Neg |
| 101           | Adm         | 7              | Conf      | Eq     | 160  | Neg | Neg | Neg |     | Conf | –     | 2560 | Neg | Neg | Neg | Neg | Neg | Neg |
| 1610          | FU          | 17             | +         | 2560   | Neg | Neg | 20 | 20   |     | Conf | –     | 2560 | Neg | Neg | Neg | Neg | Neg | Neg |
| 483           | Adm         | 6              | Comp      | –       | 2560 | 14 | Neg | 20  |     | Conf | –     | 1280 | Neg | Neg | Neg | Neg | Neg | Neg |
| 884           | FU          | 40             | +         | 2560   | 160 | 20 | Neg | 113 |     | Conf | –     | 2560 | Neg | Neg | Neg | Neg | Neg | Neg |
| 1074          | Adm         | 7              | Comp      | –       | 2560 | Neg | Neg | Neg |     | Conf | –     | 2560 | Neg | Neg | Neg | Neg | Neg | Neg |
| 1180          | FU          | 21             | +         | 2560   | 20  | 20 | Neg | 20  | Neg | Conf | –     | 2560 | Neg | Neg | Neg | Neg | Neg | Neg |
| 2053          | Adm         | 5              | Comp      | –       | 2560 | 20 | Neg | Neg |     | Conf | –     | 2560 | Neg | Neg | Neg | Neg | Neg | Neg |
| 775           | FU          | 13             | +         | 2560   | 40  | 20 | 80  |     |     | Conf | –     | 2560 | Neg | Neg | Neg | Neg | Neg | Neg |
| 5149          | Adm         | 3              | Comp      | –       | 320  | Neg | Neg | Neg |     | Conf | –     | 113  | Neg | Neg | Neg | Neg | Neg | Neg |
| 1056          | FU          | 12             | +         | 640    | Neg | Neg | 40 |     |     | Conf | –     | 640  | Neg | Neg | Neg | Neg | Neg | Neg |
| 1917          | Adm         | 1              | Unkn      | –       | 2560 | 2560 | 2560 | 2560 | 2560 | Conf | –     | 1280 | 20  | 20  | 20  | 20  | Neg | Neg |
| 1036          | FU          | 28             | +         | 2560   | 2560 | 2560 | 2560 |     |     | Conf | –     | 2560 | 20  | 20  | 20  | 20  | Neg | Neg |
| 1037          | Adm         | 7              | Unkn      | –       | 2560 | 2560 | 2560 | 453  |     | Conf | –     | 2560 | Neg | Neg | Neg | Neg | Neg | Neg |
| 1037          | FU          | 12             | +         | 2560   | 2560 | 2560 | 2560 | 320  |     | Conf | –     | 1280 | Neg | Neg | Neg | Neg | Neg | Neg |
| 1037          | Adm         | 4              | Unkn      | –       | 80   | 2560 | 226 | 320  | 226  | 20  | Conf | –     | Neg | 80  | Neg | 20  | Neg | Neg |
| 1037          | FU          | 16             | +         | 640    | 2560 | 1280 | 2560 |     |     | Conf | –     | Neg | 80  | Neg | 20  | Neg | Neg |

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-JEG IgG detection by ELISA (Euroimmun); +: positive; Eq: equivocal; −: negative.

aJEV IgG negative before depletion.
bOnly 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.

Discussion

This pilot study included a large set of well-characterized patients recruited prospectively in clinical studies, with extensive VNT for
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, 78% (26/33) 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-JE 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-JE 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 by no means novel. In 1973, Edelman and Pariyanonda39 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 WNV55 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-naive 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.
| Patient number | Days of illness | Class | Before IgG depletion, JEV IgG | JEV IgG | After IgG depletion (IgM-VNT) | NAb titre |
|----------------|----------------|-------|------------------------------|---------|-------------------------------|-----------|
| 34             | Conf           |       | −                            | −       | 160 Neg Neg Neg Neg Neg Neg Neg |           |
| 37             | 3              | Conf  | −                            | −       | 640 Neg Neg Neg Neg Neg Neg Neg |           |
| 38             | 2              | Conf  | −                            | −       | 57 Neg Neg Neg Neg Neg Neg Neg |           |
| 40             | 14             | Conf  | −                            | −       | 80 Neg Neg Neg Neg Neg Neg Neg |           |
| 44             | 4              | Conf  | −                            | −       | 57 Neg Neg Neg Neg Neg Neg Neg |           |
| 47             | 4              | Conf  | −                            | −       | 320 Neg Neg Neg Neg Neg Neg Neg |           |
| 52             | 4              | Conf  | −                            | −       | 80 Neg Neg Neg Neg Neg Neg Neg |           |
| 53             | 4              | Conf  | −                            | −       | 57 Neg Neg Neg Neg Neg Neg Neg |           |
| 59             | 4              | Conf  | −                            | −       | 320 Neg Neg Neg Neg Neg Neg Neg |           |
| 60             | 1              | Conf  | −                            | −       | 160 Neg Neg Neg Neg Neg Neg Neg |           |
| 64             | 3              | Conf  | −                            | −       | 80 Neg Neg Neg Neg Neg Neg Neg |           |
| 57             | 6              | Conf  | −                            | −       | 640 Neg Neg Neg Neg Neg Neg Neg |           |
| 66             | 8              | Conf  | −                            | −       | 40 Neg Neg Neg Neg Neg Neg Neg |           |
| 73             | 5              | Conf  | −                            | −       | 1810 Neg Neg Neg Neg Neg Neg Neg |         |
| 76             | 5              | Conf  | −                            | −       | 320 Neg Neg Neg Neg Neg Neg Neg |           |
| 87             | 4              | Conf  | −                            | −       | 57 Neg Neg Neg Neg Neg Neg Neg |           |
| 88             | 6              | Conf  | −                            | −       | 160 Neg Neg Neg Neg Neg Neg Neg |           |
| 92             | 3              | Conf  | −                            | −       | 905 Neg Neg Neg Neg Neg Neg Neg |           |
| 98             |               | Conf  | −                            | −       | 320 Neg Neg Neg Neg Neg Neg Neg |           |
| 101            |               | Conf  | −                            | −       | 1280 Neg Neg Neg Neg Neg Neg Neg |          |
| 102            |               | Conf  | −                            | −       | 640 Neg Neg Neg Neg Neg Neg Neg |           |
| 103            |               | Conf  | −                            | −       | 320 Neg Neg Neg Neg Neg Neg Neg |           |
| 104            |               | Conf  | −                            | −       | 226 Neg Neg Neg Neg Neg Neg Neg |           |
| 105            |               | Conf  | −                            | −       | 160 Neg Neg Neg Neg Neg Neg Neg |           |
| 111            |               | Conf  | −                            | −       | 452 Neg Neg Neg Neg Neg Neg Neg |           |
| 112            |               | Conf  | −                            | −       | 160 Neg Neg Neg Neg Neg Neg Neg |           |
| 127            |               | Conf  | −                            | −       | 640 Neg Neg Neg Neg Neg Neg Neg |           |
| 118            |               | Conf  | −                            | −       | 640 Neg Neg Neg Neg Neg Neg Neg |           |
| 89             | 3              | Conf  | −                            | _b      | 160 Neg Neg Neg Neg Neg Neg Neg |           |
| 97             |               | Conf  | −                            | _b      | 640 Neg Neg Neg Neg Neg Neg Neg |           |
| 94             | 3              | Conf  | −                            | _b      | 640 Neg Neg Neg Neg Neg Neg Neg |           |
| 110            |               | Conf  | −                            | _b      | 160 Neg Neg Neg Neg Neg Neg Neg |           |
| 121            |               | Conf  | −                            | _b      | 160 Neg Neg Neg Neg Neg Neg Neg |           |
| 54             | 3              | Conf  | −                            | −       | 57 Neg Neg Neg Neg Neg Neg Neg |           |
| 128            |               | Conf  | Eq                           | −       | 640 Neg Neg Neg Neg Neg Neg Neg |           |
| 51             | 5              | Conf  | Eq                           | −       | 905 Neg Neg Neg Neg Neg Neg Neg |           |
| 33             |               | Conf  | Eq                           | −       | 452 Neg Neg Neg Neg Neg Neg Neg |           |
| 58             | 5              | Conf  | Eq                           | −       | 2560 Neg Neg Neg Neg Neg Neg Neg |         |
| 62             | 6              | Conf  | Eq                           | −       | 226 20 Neg Neg Neg Neg Neg Neg Neg |         |
| 35             | 4              | Conf  | +                            | −       | 160 Neg Neg Neg Neg Neg Neg Neg |           |
| 36             |               | Conf  | +                            | −       | 40 Neg Neg Neg Neg Neg Neg Neg |           |
| 41             | 4              | Conf  | +                            | −       | 320 Neg Neg Neg Neg Neg Neg Neg |           |
| 65             | 13             | Conf  | +                            | −       | 80 14 Neg Neg Neg Neg Neg Neg Neg |         |
| 67             | 4              | Conf  | +                            | −       | 1280 Neg Neg Neg Neg Neg Neg Neg |         |
| 68             | 6              | Conf  | +                            | −       | 2560 Neg Neg Neg Neg Neg Neg Neg |         |
| 69             | 5              | Conf  | +                            | −       | 452 Neg Neg Neg Neg Neg Neg Neg |           |
| 70             | 6              | Conf  | +                            | −       | 640 Neg Neg Neg Neg Neg Neg Neg |           |
| 71             | 8              | Conf  | +                            | −       | 640 Neg Neg Neg Neg Neg Neg Neg |           |
| 74             | 4              | Conf  | +                            | −       | 226 Neg Neg Neg Neg Neg Neg Neg |           |
## Table 4. Continued

| Patient number | Days of illness | Class | Before IgG depletion, JEV IgG | JEVD1 IgG | JEVD2 IgG | JEVD3 IgG | JEVD4 IgG | WN Ab titre |
|----------------|----------------|-------|-------------------------------|-----------|-----------|-----------|-----------|-------------|
| 75             | 14             | Conf  | −                             | −         | 905       | Neg       | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 79             | 5              | Conf  | +                             | −         | 640       | Neg       | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 80             | 7              | Conf  | +                             | −         | 226       | Neg       | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 81             | 6              | Conf  | +                             | −         | 640       | Neg       | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 82             | 6              | Conf  | +                             | −         | 905       | Neg       | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 86             |                | Conf  | +                             | −         | 2560      | Neg       | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 91             | 7              | Conf  | +                             | −         | 1280      | Neg       | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 95             | 4              | Conf  | +                             | −         | 320       | Neg       | Neg       | Neg        | Neg         | 40          | Neg         | Neg         | Neg         |
| 99             |                | Conf  | +                             | −         | 113       | Neg       | Neg       | Neg        | 20          | Neg         | Neg         | Neg         | Neg         |
| 108            |                | Conf  | +                             | −         | 320       | Neg       | Neg       | Neg        | 40          | Neg         | Neg         | Neg         | Neg         |
| 109            |                | Conf  | +                             | −         | 113       | Neg       | Neg       | Neg        | 20          | Neg         | Neg         | Neg         | Neg         |
| 116            |                | Conf  | +                             | −         | 113       | Neg       | Neg       | Neg        | 20          | Neg         | Neg         | Neg         | Neg         |
| 122            |                | Conf  | +                             | −         | 1280      | Neg       | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 125            |                | Conf  | +                             | −         | 160       | Neg       | Neg       | 14         | Neg         | Neg         | Neg         | Neg         | Neg         |
| 31             |                | Comp  | −                             | −         | 226       | Neg       | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 32             |                | Comp  | −                             | −         | 226       | Neg       | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 45             | 10             | Comp  | −                             | −         | 80        | Neg       | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 56             | 6              | Unkn  | +                             | −         | 80        | Neg       | 40        | 20         | Neg         | Neg         | Neg         | Neg         | Neg         |
| 77             | 6              | Unkn  | +                             | −         | 80        | Neg       | 28        | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 85             |                | Unkn  | +                             | −         | 320       | 98        | 160       | 57         | 20          | Neg         | Neg         | Neg         | Neg         |
| 78             | 4              | Unkn  | +                             | −         | 160       | Neg       | Neg       | Neg        | 80          | Neg         | Neg         | Neg         | Neg         |
| 39             | 3              | Neg   | −                             | −         | Neg       | Neg       | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 83             | 5              | Neg   | −                             | −         | Neg       | Neg       | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 48             | 3              | Neg   | −                             | −         | Neg       | Neg       | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 61             | 14             | Neg   | −                             | −         | Neg       | 14        | 14        | Neg        | 14          | Neg         | Neg         | Neg         | Neg         |
| 49             | 10             | Neg   | −                             | −         | Neg       | 14        | Neg       | Neg        | Neg         | Neg         | Neg         | Neg         | Neg         |
| 120            |                | Neg   | −                             | −         | 20        | 40        | 20        | 14         | Neg         | Neg         | Neg         | 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.

*Only one replicate tested or interpretable, the other samples were tested in duplicate.
*J EV IgG negative before depletion.
*Maximum dilution tested.
*Test not performed.

## Supplementary data

Supplementary data are available at *Transactions* online.

## 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 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.

## Acknowledgements

We are very grateful to the patients and to Bounthaphany Bounsouali, 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 Hosp.
tal. We also thank Bouonnak Sayanasongkham, the former Director of the Department of Healthcare and Rehabilitation, Ministry of Health, and Bounkong Syhavong, Minister of Health, Laos PDR for their very kind help and support. We thank the stakeholders of the SEAE project, members of the Unité des Virus Emergents (Christine Isnard and Camille Plaidoi) and the CNR des Arbovirus (Patrick Grolier, Gilda Grard, Isabelle Leparc-Goffart and Mathilde Galla). We also thank Rodrigo Cachay, Eduardo Gotozuo 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 Crabot, Philippe Dussart, Marc Elloit, Magali Herrant, Nguyen Hien, Chau Su Hlaing, Jerome Honnorat, Tran Thai 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 Pola, Bruno Rosset, Ky Santy, Heng Sotthy, Arnaud Tarantola, Nguyen Thi Thu Huyn, Htay Htay Tin, Ommar Sere Ting, Pham Nhat An, Dang Duc Anh, Pascal Bonnet, Kimrong Bun, Danay Channamman, Viengmon Davong, Patrice Devre, Jean-Francois Defraissy, Christian Devaux, Anousone Douangnoungvong, Veasna Duong, Benoit Durand, Chanreosmekmy Eng, Catherine Ferranti, Didier Fontenille, Lukas Hofner, Le Thanh Hoi, Do Thu Huong, Marc Jouan, May July, Magali Lago, Jean-Paul Moatti, Bernadette Murgaue, Xin Yi Guo, Meng-Heng Oum, Khounsaudophone Phakhounthong, Anh Tuan Pham, Do Quyen, Malee Seepheonee, Maud Seguy, Bountoy Sibounsounuang, Kanharith Min, Chao Thair, Winn Thein, Phung Bich Thuy, Herve 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) 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 (SIDIJISI 103488).

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

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