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**Original Article**

# Dengue Associated Acute Encephalitis Syndrome Cases in Son La Province, Vietnam in 2014

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**SUMMARY:** Acute encephalitis syndrome (AES) is associated with high morbidity and mortality, and affects both children and adults. The main etiologic agent is Japanese encephalitis virus (JEV); however, there are also reports of Dengue virus (DENV) encephalitis. The objectives of this study were to determine the proportion of patients with encephalitis due to JEV during the 2014 outbreak in Son La Province in Vietnam and to explore the association of DENV in non-JEV viral encephalitis cases. Of 90 patients, 6 (6.7%) were positive for anti-JEV immunoglobulin M (IgM), 5 (5.6%) were positive for anti-DENV IgM, 30 (33.3%) were positive for both anti-JEV and anti-DENV IgM, and 56 (62.2%) were positive for flavivirus immunoglobulin G (IgG). In 5 patients with AES, who had positive anti-DENV IgM results in at least one of the paired serum samples, DENV was confirmed by neutralization testing. The incidence of JEV infection was high. There is still a need to maintain and strengthen the national JEV immunization program. This noticeable occurrence of DENV infection was not reported in Son La Province in 2013–2014. Our data suggested that in addition to JEV, DENV was also a causative agent of AES in 2014 in Son La Province, and this finding also confirmed the local occurrence of DENV infection.

## INTRODUCTION

Acute encephalitis syndrome (AES) is a major public health problem in Asia. AES is defined as the acute onset of fever and a change in mental status (including symptoms such as confusion, disorientation, coma, or aphasia) and/or new onset of seizures (excluding simple febrile convulsion) in a person of any age at any time of year (1). Among the AES cases, the main suspected etiological agent is Japanese encephalitis virus (JEV) based on clinical features and epidemiological factors; however, encephalitis due to other most common viruses such as enteroviruses (EV), dengue virus (DENV), and herpes simplex virus (HSV) has also been reported in Vietnam (2,3). Japanese encephalitis (JE) is a significant public health problem in Vietnam with epidemics having been reported since the late 1960s (4-7).

Annual incidence of AES in Vietnam had ranged from 1 to 8 cases per 100,000 population from 1985 to 1993 (6). In 1997, JE vaccine was introduced in high-risk areas in northern Vietnam and subsequently to its other parts (8). From 1998 to 2007, the mean annual incidence of AES was 2.4 cases per 100,000 population (9). A slightly decreasing trend of incidence of encephalitis was noted after the introduction of JE vaccine; in addition, no JE epidemics, which were similar to those prior to the introduction of the vaccine, had been reported (9). In most parts of the country, immunization with JE vaccine covers children aged 1–5 years (9).

In 2014, northern Vietnam reported 741 JE cases in 25 of 28 provinces, with 35 deaths in 8 provinces, which was an increase of 38% compared with the 2013

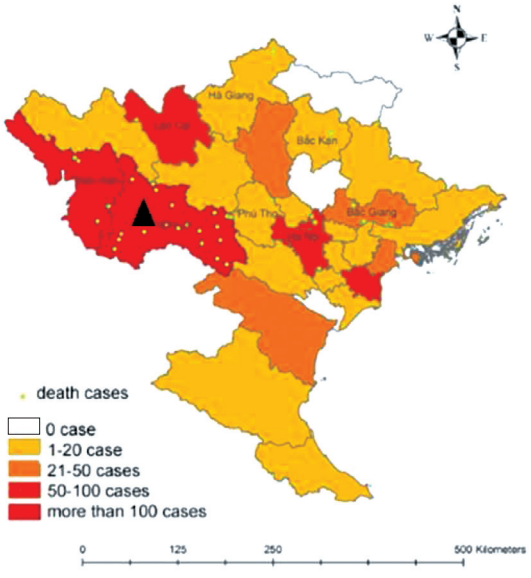


Fig. 1. (Color online) No. of acute encephalitis syndrome cases and death cases in northern Vietnam in 2014 and location of Son La hospital (triangle) in Son La Province.

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cases. Usually, the northwest regions, which include Son La Province, have a high incidence of AES (>6.0 cases per 100,000 population) compared to other parts of the country despite the introduction of JE vaccine (9). However, there are no reports of DENV outbreaks in Son La Province. The Department of Epidemiology, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam, provided the number of AES cases and deaths in northern Vietnam in 2014 (Fig. 1). Between June and August 2014, a high number of AES-suspected cases were detected, indicating an outbreak. The majority of the cases and death reports came from Son La Province (i.e., 201 cases with 21 deaths). It represented a significant outbreak since the introduction of JE vaccine in the country.

In this study, using samples from Son La Province, Vietnam, we aimed to describe the proportion of encephalitic patients due to JEV during the reported JE outbreak and to evaluate whether DENV was also a causative agent.

## MATERIALS AND METHODS

**Patients:** Patients with clinical features suggestive of AES admitted to Son La hospital in Son La Province from June to September 2014 were included in the study. Patient inclusion criteria were based on the World Health Organization (WHO) case definition of AES (10). Informed written consent was obtained from the patients/guardians prior to the study. Acute and/or convalescent samples were collected from each patient.

**DENV and JEV IgM capture ELISA:** Serum samples were subjected to an in-house anti-JEV IgM and anti-DENV IgM capture enzyme-linked immunosorbent assays (ELISAs). In-house IgM-capture ELISA was carried out using the protocol described by Bundo and Igarashi (11) and our previous report (12), with minor modifications. Both ELISAs were performed with the same steps and reagents except for the assay antigen. Microplates (96-well) were coated with 100 µL (5.5 µg/100 µL) of anti-human IgM goat IgG (Cappel ICN Pharmaceuticals, USA) and incubated at 37°C for 1 h. Wells were blocked with Block Ace (UK-B 80, Yukijirushi, Sapporo, Japan) and incubated at room temperature (RT) for 1 h. After incubation, wells were washed three times with PBS containing 0.05% Tween 20 (PBS-T). Test samples and controls (positive and negative) were diluted 1:100 in PBS-T and 100 µL volumes were distributed into duplicate wells. The plate was incubated at 37°C for 1 h and then washed as described above. JEV (strain JaOArS982) antigen (128 ELISA units) was added (100 µL/well), then the plate was incubated for 1 h at 37°C. For the DENV IgM ELISA, tetravalent DENV antigen was used. After washing, 1:2,500 dilution of HRP-conjugated anti-flavivirus mouse monoclonal antibody was added (100 µL/well). The plate was washed and 100 µL of *o*-Phenylenediamine dihydrochloride (OPD) substrate was added per well, then the plate was kept in the dark at room temperature for 1 h. To terminate the reaction, 100 µL of 1 N sulfuric acid was added to each well, and the optical density (OD) was read at 492 nm (Multiscan JX). A positive control at $OD_{492}$ (or sample)/negative control at $OD_{492}$ ratio of $\geq 2.0$ was considered positive.

**Flavivirus IgG indirect ELISA:** The in-house flavivirus IgG indirect ELISA was carried out following the protocol described previously (12). The protocol for flavivirus IgG detection shared similar procedures (volumes of solutions, incubation time and washing steps) with the IgM detection method except in the order of adding the reactants or reagents and the concentrations used. The following were added to each well in successive manner: purified JEV (strain JaOArS982) antigen, Block Ace, serum samples at 1:1,000 dilution, HRP-conjugated anti-human IgG (American Qualex, San Clemente, CA, USA) at 1:30,000 dilution and OPD substrate. A standard curve was prepared using the $OD_{492}$ values of the DENV-positive control serum sample starting with a 1,000-fold dilution, followed by a serial two fold dilutions. A sample titer equal to or greater than 1:3,000 was considered positive.

**Plaque reduction neutralization test:** To confirm the cause of infection among the AES patients with serum samples that gave negative or low positive results to anti-JEV IgM ELISA and a positive result to anti-DENV IgM in at least one of the paired serum samples, their samples were checked for the ability to neutralize DENV and JEV by 50% plaque reduction neutralization test ($PRNT_{50}$). We followed the PRNT protocol by Moi et al. (13) with a slight modification. In this procedure, patient serum samples were heat inactivated at 56°C for 30 min prior to the assay. Serum was serially diluted two fold starting from 1:10. However, in samples with inadequate volume, the dilutions from 1:20 and 1:40 were used. The virus strains used for neutralization tests were as follows: DENV-1, M-120 strain; DENV-2, M-58 strain; DENV-3, SLMC50 strain; and DENV-4, SLMC318 and JEV-JaOArS982 strains. For each dilution, 150 µL of the serum sample was mixed with an equal volume of virus infected tissue culture fluid that contained 40 plaque-forming units of a specific virus serotype. The mixture was incubated for 1 h at 37°C for a virus-antibody neutralization reaction, then 150 µL of each mixture was used to inoculate Fc gamma receptor (FcγR)-expressing BHK cell monolayer in a 24-well plate. After incubation at 37°C for 1 h , the infected cells were overlaid with 500 µL of EMEM (Nissui Pharmaceutical,Tokyo, Japan) containing 2% FCS and 1% methylcellulose (Wako Pure Chemical Industries,Osaka, Japan). The plates were then incubated at 37°C for 3–5 days based on the dengue serotype used. The plate was washed with PBS to remove methylcellulose, added to 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries), and cells were stained with 0.1% crystal violet in 10% ethanol. The reciprocal of the endpoint serum dilution that provided a 50%, or greater, reduction in the mean number of plaque relative to the control wells that contained no serum was considered the $PRNT_{50}$ titer.

**Ethical issue:** This study was approved by the Institutional Review Board of NIHE, Vietnam (No.15-HDD, January 18, 2011).

## RESULTS

**Serological profiles of AES patients:** In this study, there were 90 patients (49 male and 41 female patients) with AES manifestations. All patients were residents of

Son La Province and aged 0.9–77 years (mean age = 22 years). Only 4 patients had a history of JE vaccination. A total of 107 serum samples were collected: only acute samples came from 22 patients, paired acute and convalescent samples from 16 patients, and only convalescent samples from 52 patients. Based on the available serum samples, the number (%) of patients positive for specific antibodies were as follows: 6 (6.7%) for anti-JEV IgM only, 5 (5.6%) for anti-DENV IgM only, and 30 (33.3%) for both anti-JEV and anti-DENV IgM. A total of 41 of the 90 patients (45.5%) were positive for at least one of these antibodies, and 49 (54.4%) were negative for both. The distribution of 41 AES patients (according to age group [years]) positive for either or both antibodies showed that the age group of 1-10 years had the highest number of positives (Fig. 2). Results of the flavivirus IgG assay showed that 56 (62.2%) were positive for flavivirus IgG and 34 (37.7%) were negative. All the vaccinated patients had positive titers for flavivirus IgG.

**Characterization and confirmation of DENV infection among DENV IgM positive cases:** As mentioned above, 5 patients had positive results for anti-DENV IgM ELISA, but had negative results for anti-JEV IgM ELISA. The clinical symptoms of 5 patients included high fever (>38°C), headache, vomiting, vague symptom, neck stiffness, convulsion, change of mental state and low Glasgow Coma Scale. Laboratory analysis of blood and cerebrospinal fluid (CSF), which was available only in 3 of the 5 patients (Table 1), showed that 2 had platelet counts below the normal level but also had levels of high aspartate transaminase (AST) and alanine transaminase (ALT). Gram staining of CSF and bacteria isolation was negative for the 3 patients. All 5 patients had primary flavivirus infection based on the flavivirus IgG indirect ELISA. DENV serotype-specific and JEV neutralization tests ($PRNT_{50}$) confirmed that all 5 patients from among these unknown AES cases had DENV infection (Table 2). The highest neutralizing titer was found against DENV-1 in 4 patients and against DENV-3 in one patient. Low neutralizing levels were detected against JEV and other dengue serotypes.

**Corroboration of DENV or JEV infection among patients positive for JEV IgM only or both JEV and DENV IgM:** It was previously mentioned that there were 6 AES patients positive only to anti-JEV IgM, and the results of JEV and DENV $PRNT_{50}$ from testing of their convalescent serum samples revealed higher neutralization titer against JEV compared to DENV, thus confirming JEV infection (Table 2). DENV neutralizing antibodies were detected, but the levels were low.

There were 30 AES patients positive for both anti-JEV IgM and anti-DENV IgM. Their serum samples had higher JEV IgM P/N ratio than that of DENV, and only 17 had enough volume of serum for determination of $PRNT_{50}$; these results showed that 14 patients had higher neutralization titer against JEV compared to that against DENV, thus confirming JEV infection (Table 2). The other 3 patients had the same level of neutralizing antibodies against both DENV and JEV.

Table 1. Laboratory analysis of 3 dengue suspected AES patients

| | Patient ID | | |
|---|---|---|---|
| Hemogram | 6,113 | 6,115 | 5,953 |
| WBC ($\times 10^3$/μL) | 5.7 | 11.9 | 21.7 |
| Hb (g/dL) | 16 | 11.9 | 12.2 |
| Hct (%) | 48.5 | 38.7 | 37.2 |
| PLT ($\times 10^3$/μL) | 80 | 117 | 602 |
| AST (U/L) | 86 | 78 | 35 |
| ALT (U/L) | 103 | 94 | 29 |
| CSF observation | | | |
| WBC (cells/μL) | 5 | 8 | 40 |
| protein (g/L) | 0.8 | 0.4 | 1.2 |
| Glucose (mmol/L) | 2.8 | 5.2 | 5.6 |
| Gram stain | neg | neg | neg |
| Bacterial isolation | neg | neg | neg |

WBC, white blood cells; Hb, Hemoglobin; Hct, Hematocrit; PLT, platelet; AST, aspartate transaminase; ALT, alanine transaminase; neg, negative.

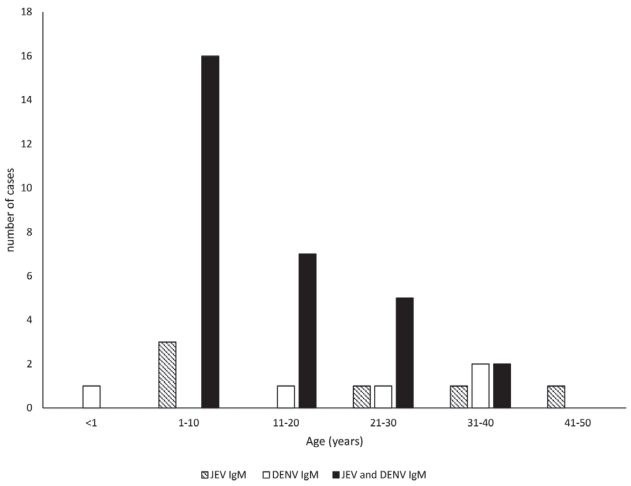


Fig. 2. Age distribution of patients positive only to anti-Japanese encephalitis virus IgM or anti-Dengue virus IgM or positive to both antibodies.

## DISCUSSION

Our study showed that of 90 AES patients, 36 (44.0%) were positive for the presence of anti-JEV IgM, 5 (5.6%) were positive for anti-DENV IgM only and 56 were positive for flavivirus IgG. Those positive for anti-JEV IgM included the 6 patients who were only positive for anti-JEV IgM and 30 patients positive for both anti-JEV IgM. Those that were positive for both antibodies had JEV IgM P/N ratio higher than that of DENV. The incidence of JEV was more common in northern Vietnam than in central and southern Vietnam (9). According to AES surveillance guidelines, a clinical case of AES with JEV-specific IgM antibody positivity in a single sample of CSF or serum as detected by an IgM-capture ELISA is considered a laboratory-confirmed case (10). Therefore, the AES patients in the present study were confirmed to have JE. For reliable serological diagnosis of JE, samples should be collected at least 10 days after the onset of patient's illness (10). For microbiological confirmation, the better sample is CSF. In this study, most of the samples were obtained during the convalescent phase. CSF samples were not available from all the patients. Other AES agents, including bacteria and parasites, have been suggested as causative agents of AES for patients negative for anti-flavivirus antibodies (2,14-17). In Vietnam, anti-JEV

IgM positivity among AES patients varies from 17 to 71% (9).

DENV infection, another mosquito borne disease in humans, has become an important epidemic disease in Vietnam. Neurological manifestations of dengue infection have been reported in 0.5-6% of DENV hemorrhagic fever patients (17-19). Our data presented that 5.6% had anti-DENV IgM and the presence of this antibody indicated a recent DENV infection among AES patients. The dengue IgM-positive samples were confirmed by PRNT test (DENV and JEV neutralization tests). The FcγR-expressing BHK cells were used to determine neutralizing antibody titers because this could better reflect protective immunity where the principal target cells of DENV are Fc (gamma) R-expressing cells, such as monocytes (20). Furthermore, the highest neutralization titer among 5 AES patients was detected against DENV-1 and DENV-3. We did not measure NS1 antigen test in these patients due to inadequate amount of serum. In addition, we could not confirm the infecting serotypes by virus isolation because the serum samples were stored at temperatures that are below optimal levels for virus isolation. In the absence of isolated virus, according to the previously published criteria of monotypic pattern of anti-DENV neutralizing antibodies (21,22), the DENV serotype with the highest neutralization titer was assumed to be the infecting serotype. Thus, from among the 5 patients, 3 were infected with DENV-1, one was infected with DENV-3; however, it was difficult to conclude the infecting serotype for one patient (sample ID-6113-II), because the antibody titer against DENV-1 and DENV-3 were very similar.

In the present study, 6 patients positive for only anti-JEV IgM were confirmed to have JEV infection using the PRNT test. There were 30 patients positive for both anti-JEV and anti-DENV IgM. PRNT tests of available serum samples from 17 of these patients showed that most of them (14 patients) had JEV infection. As for

Table 2. Characterization and confirmation of DENV or JEV infection among the anti-DENV IgM only, JEV IgM only and both JEV and DENV IgM positive cases

| No. | Sample ID | Sex | Age (yr) | JEV IgM[2)] | DENV IgM[2)] | Flavivirus IgG[3)] | Neutralization titer ($PRNT_{50}$) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | D1 | D2 | D3 | D4 | JEV | Diagnostic Interpretation |
| JEV IgM (–), DENV IgM (+) | | | | | | | | | | | | |
| 1 | 5915-I[1)] | M | 39 | 1.7 | 4.0 | 11,029 | 20 | 20 | 40 | 20 | 80 | DENV-1 |
| | 5915-II[1)] | | | 1.7 | 3.7 | 15,554 | 160 | 20 | 40 | 20 | 40 | |
| 2 | 5952-II | F | 36 | 0.9 | 2.9 | 4,148 | 160 | < 20 | < 20[4)] | < 20 | 40 | DENV-1 |
| 3 | 5953-I | M | 27 | 0.3 | 1.1 | 1,701 | < 10 | < 10 | < 10 | < 10 | < 10 | DENV-3 |
| | 5953-II | | | 0.8 | 2.4 | 3,614 | < 40 | < 40[4)] | 40 | < 40 | < 40 | |
| 4 | 6113-II | M | 0.91 | 0.7 | 2.0 | 769 | 20 | < 10 | 10 | < 10 | < 10 | DENV |
| 5 | 6115-II | F | 15 | 3 | 6.5 | 7,355 | 10 | < 10 | < 10 | < 10 | < 10 | DENV-1 |
| JEV IgM (+), DENV IgM (–)[5)] | | | | | | | | | | | | |
| 1 | 5881 | M | 5 | 8.1 | 1.9 | 69,128 | < 20 | 20 | 20 | 20 | > 2,560 | JEV |
| 2 | 5897 | M | 4 | 8.6 | 1.8 | 350 | 20 | 20 | < 20 | 20 | 160 | JEV |
| 3 | 5903 | M | 42 | 2.2 | 0.8 | 5,520 | < 20 | < 20 | < 20 | < 20 | 20 | JEV |
| 4 | 5914 | F | 22 | 2.3 | 0.8 | 12,156 | 20 | < 20 | 20 | 20 | 80 | JEV |
| 5 | 5959 | F | 6 | 11.7 | 1.9 | 3,021 | 40 | < 20 | 40 | 40 | 160 | JEV |
| 6 | 6052 | F | 32 | 2.5 | 1.1 | 23,992 | < 20 | < 20 | 20 | < 20 | 40 | JEV |
| JEV IgM (+), DENV IgM (+)[5)] | | | | | | | | | | | | |
| 1 | 5856 | F | 6 | 13.9 | 7.8 | 4,092 | < 20 | < 20 | < 20 | < 20 | 40 | JEV |
| 2 | 5857 | F | 5 | 15.8 | 7 | 3,614 | 20 | < 20 | 20 | 20 | 320 | JEV |
| 3 | 5858 | F | 17 | 8 | 2.4 | 67,325 | < 20 | < 20 | 20 | < 20 | 160 | JEV |
| 4 | 5859 | M | 10 | 15.9 | 7.9 | 15,731 | < 20 | < 20 | 20 | < 20 | 160 | JEV |
| 5 | 5860 | M | 3 | 14.4 | 4.3 | 127,092 | 20 | < 20 | < 20 | < 20 | 160 | JEV |
| 6 | 5861 | M | 10 | 13.8 | 8.1 | 63,424 | 20 | < 20 | < 20 | < 20 | 640 | JEV |
| 7 | 5864 | M | 16 | 13.9 | 5.9 | 44,657 | < 20 | < 20 | 20 | < 20 | 40 | JEV |
| 8 | 5865 | M | 10 | 13.8 | 5.3 | 51,404 | 20 | < 20 | 20 | 20 | > 2,560 | JEV |
| 9 | 5880 | M | 1 | 13.3 | 6.1 | 63,460 | 20 | < 20 | < 20 | < 20 | 320 | JEV |
| 10 | 5882 | F | 2 | 14.9 | 4.6 | 23,572 | < 20 | < 20 | < 20 | 40 | 160 | JEV |
| 11 | 5898 | F | 2 | 15.3 | 11.5 | 11,234 | < 20 | < 20 | < 20 | < 20 | 40 | JEV |
| 12 | 5956 | F | 11 | 11.6 | 3.8 | 4,114 | 20 | < 20 | < 20 | < 20 | 20 | JEV |
| 13 | 6041 | F | 10 | 15.3 | 12.5 | 10,081 | 20 | < 20 | 40 | 20 | 640 | JEV |
| 14 | 6048 | M | 4 | 10.8 | 2.6 | 913 | 20 | < 20 | < 20 | 20 | 640 | JEV |
| 15 | 6049 | F | 22 | 10.8 | 4 | 140,138 | < 20 | < 20 | 20 | < 20 | 20 | JEV |
| 16 | 6051 | M | 21 | 14.7 | 2.4 | 4,176 | 20 | < 20 | < 20 | < 20 | 20 | JEV |
| 17 | 6114 | F | 20 | 6.9 | 2 | 359 | < 20 | < 20 | 20 | < 20 | 640 | JEV |

[1)]: I, Acute phase serum; II, convalescent phase serum.
[2)]: Patients serum samples; positive to JEV and DENV IgM (P/N ratio ≥ 2).
[3)]: Positive for flavivirus infection if IgG titer ≥ 3,000; with secondary infection if IgG titer ≥ 52,000 and with primary infection if IgG titer < 52,000.
[4)]: Starting dilution- either 1:20 or 1:40 due to inadequate serum volume.
[5)]: All samples were convalescent phase serum.

the remaining 3 patients, it was difficult to conclude whether infection was due to DENV or JEV, because the antibody neutralization titers were similar. However, anti-JEV IgM was higher than anti-DENV IgM; therefore, it could be due to JEV infection. In addition to a high antibody titer against one DENV serotype, low neutralizing antibody titer against other serotypes was observed in some serum samples. This finding may be due to the cross reactivity of the antibodies to other DENV serotypes. In the patients with confirmed DENV infection, the lower JEV neutralization titer may be related to previous infection or cross reactivity of the antibodies to flavivirus, which includes JEV. Other studies have also noted that the antibodies from dengue-infected patients are cross reactive against other flaviviruses (23,24).

Based on the data of the National Institute of Hygiene and Epidemiology in Vietnam, no DENV outbreak was reported in Son La Province during 2013–2014. However, our data showed the existence of dengue infection that was associated with AES during this period. Co-existing DENV infection with other unknown encephalitis virus infection could have also possibly occurred within this span of time. A previous study had identified that in southern Vietnam, JEV, EV, and DENV were the most common etiologies of infection for children with acute encephalitis, accounting for 26%, 9.3%, and 4.6% of cases, respectively (2). In this study, 95.6% (86/90) were not immunized against JE because most of them were of the age not covered when the JE immunization program started. JE immunization has covered >90% of target population in Vietnam (8), but the presence of AES cases shows there is still a need to strengthen the national immunization program.

In conclusion, we identified the incidence of JEV and DENV infection among AES patients in Son La Province. We also found that dengue was one of the causative agents in AES cases in Son La Province. Our results also suggest a possible previous DENV outbreak in Son La Province.

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**Conflict of interest** None to declare.

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