ISOLATION OF DENGUE SEROTYPE 3 VIRUS FROM THE CEREBROSPINAL FLUID OF AN ENCEPHALITIS PATIENT IN HAI PHONG, VIETNAM IN 2013

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Abstract

Dengue encephalitis (DE) is characterized as unusual presentation of dengue infection. Despite the reports that DE accounts for only 1% to 5% of dengue cases, this disease tends to be increasingly reported to threaten global human health throughout dengue endemic areas particularly in Southeast Asia. The molecular information of clinically characterized, neurotropic dengue virus (DENV) in human beings is extremely scarce despite it playing an important role in deciphering the pathogenesis of dengue-related neurological cases. Here we report a case of DE caused by DENV3 genotype III in a male patient with atypical symptoms of DENV infection in Hai Phong, Vietnam in 2013. The virus isolated from the cerebrospinal fluid of this case-patient was closely related to DENV3 genotype III strains isolated from serum of two other patients, who manifested classical dengue in the same year and residing in the same area as the case-patient. It is noteworthy to mention that in 2013, DENV3 genotype III was detected for the first time in Vietnam.

1. Why this case is important

Dengue virus (DENV) is a member of the family *Flaviviridae* consisting of neurotropic viruses such as Japanese encephalitis virus, West Nile virus, St Louis encephalitis virus and Murray Valley encephalitis virus. Unlike these pathogens, DENV rarely causes neurologic symptoms *(1)*. The early evidence of central nervous system (CNS) manifestations associated with DENV infection was reported in several worldwide dengue outbreaks, predominantly in endemic countries of Southeast Asia *(1-6)*. These unusual presentations have been classified as severe dengue cases *(7)* , however, the neuropathogenesis of DENV infection is still poorly understood *(8)*. Previously, neurologic manifestations were considered as the consequences of an encephalopathy secondary to prolonged shock, hyponatraemia, hepatic failure or intracranial bleeding rather than encephalitis because of the failure to demonstrate the presence of DENV in the CNS *(9, 10)*. Since the 1990s, the concept of DENV neurotropism was noticed and the number of reports on dengue patients with virus isolation from CSF or brain tissue has risen *(10, 11)*. We hereby report a case of encephalitis caused by DENV3 in a male patient with atypical symptoms of DENV infection in Hai Phong, Vietnam. Virus isolation was achieved from CSF specimen. The complete envelope (E) sequence of CSF-derived DENV3 isolated from this patient was explored to define phylogenetic relationship with other DENV3 strains isolated from Hai Phong, Vietnam and from neighboring countries. We noted that DENV3 genotype III was seen for the first time in Vietnam in 2013.

2. Case description

A 25-year-old male, who worked as an accountant in Hai Phong, Vietnam, was admitted to Viet Tiep's hospital on day 1 of the onset of illness due to high-grade fever and severe headache. There was no myalgia, joint pain, diarrhea, skin and mucosal bleeding. The

patient did not have any past medical records indicating of DENV infection prior to this event. On admission, general examination did not reveal any significant abnormalities. Neurological examination did not show any focal neurological deficits apart from neck stiffness. Laboratory tests showed normal range in hemogram with slight increase in C – reactive protein (Table 1). CSF analysis revealed 10 white blood cells (WBC)/mm^3 but there was no information relating to protein, sugar and lactate (Table 1). A provisional diagnosis of suspected meningoencephalitis was made and the patient was given an empiric antibiotic treatment (Rocephin – the 3^{rd} generation cephalosporin antibiotic) in view of the possibility of bacterial infection. Despite this treatment, his symptoms were not improved, therefore, he was transferred to the referral hospital on day 3 of disease onset. In this hospital, leukopenia was noted in the laboratory tests; CSF analysis revealed 8 WBC/mm^3 and slight increase in protein concentration (Table 1). Treatment was continued with the 3^{rd} generation of cephalosporin antibiotic (Ceftriaxon) and analgesic. Occasionally, the patient was prescribed with oral sedative (Seduxen) to control the irritation due to severe headache. On day 6 of disease onset, hemogram showed continuous leukopenia associated with mild thrombocytopenia and dengue serology (SD BIOLINE Dengue Duo kit) was positive for anti-dengue IgM and IgG antibodies. Additionally, the patient started to feel improvement from the symptoms of fever and headache simultaneous with the appearance of maculopapular rash. On day 9 of disease onset, hemogram indicated a normalization of leukocyte and platelet counts (Table 1). The patient was discharged after 9 days of hospitalization without any neurological sequelae.

Following these results, a CSF specimen of this patient collected on day 1 of disease onset in Viet tiep's hospital was retrospectively analyzed. In this CSF sample, although the presence of anti-dengue IgM antibodies was negative by the in-house ELISA test, the presence of DENV RNA was confirmed by reverse transcriptase polymerase chain reaction

(RT-PCR) and DENV3 was successfully isolated (Table 2). RNA sequence of this virus strain was read by Ion Proton and 3100 Avant genetic analyzer (Life Technologies). Phylogenetic analysis was conducted using the envelop protein coding region sequence with the two other serum-derived DENV3 strains isolated in Hai Phong, Vietnam in 2013 and with other DENV3 strains worldwide.

3. Other similar and contrasting cases in literature

Following the earliest evidence of CNS involvement associated with DENV infection since 18th century *(12)*, neurological cases due to DENV3 have been increasingly reported in recent years worldwide *(1, 5, 10, 13, 14)*. In the southern part of Vietnam, there was one study that described the phylogenetic relationship between CSF-derived DENV2 and serum-derived DENV2 from the same DE patient *(14)*, however, there are no published data on DENV3 genotype III infecting a patient with neurological manifestation.

4. Discussion

Dengue encephalitis has been noticed as an unusual presentation with the prevalence being estimated to occur in 1% to 5% of dengue cases *(8)*. Neurologic manifestations caused by DENV can occur in a patient at the age of few months to 79 years and more frequently in children *(8)*. The median time for onset of CNS symptoms was reported to be 3 to 7 days from the onset of fever *(1, 4)*. Interestingly, *Soares* et al (2006) reported the existence of CNS involvement for longer period ranging between 4 to 30 days with a median time of 12 days after the onset of the fever among 13 patients aged 11 to 79 years *(15)*. Surprisingly, the presented case showed CNS symptoms on day 1 of the onset of fever.

In comparison to other dengue serotypes, DENV2 and DENV3 tend to have the highest propensity to neurological complications whether in primary or secondary infections *(1, 4)*.

Despite encephalitis being recognized as the most common presentation among patients suffering neurological manifestations due to DENV infection, only 50% of these patients present the typical features of DENV infection *(8)*; thus the existence of underestimated cases. Our patient from the beginning did not have any typical symptoms of dengue until the hemogram from the referral hospital suggested DENV infection. However, CSF analysis indicated viral infection at the early timing of disease course. Retrospective analysis of a CSF specimen drawn on day 1 of fever onset was negative for anti-dengue IgM antibody by in-house ELISA test. This result is acceptable because in serum specimens, IgM antibodies are detectable by day 3-5 from the onset of illness in 50% of patients, increasing to 80% by day 5 and 99% by day 10 during primary infection and significantly lower in secondary infection *(7)*. Furthermore, data on ELISA test for dengue specific IgM antibodies revealed 46% sensitivity in CSF of patients with neurological disorders, however, the absence of these antibodies does not exclude dengue as the causative agent of CNS abnormalities *(15).* The day for collecting CSF sample from this patient in the 1st hospital, i.e. day 1 from the onset of the disease was too early to detect anti-dengue IgM antibodies in serum or in CSF, but it was proper timing for virus isolation.

To isolate the virus, CSF specimen (10ul) was inoculated onto C6/36 mosquito cells and Vero cells (African green monkey kidney). Cells were observed daily for cytopathic effect (CPE) for seven days, RNA was extracted from culture fluid on day seven by using QIAamp Viral RNA mini kit (QIAGEN) and the presence of DENV3 RNA was confirmed by RT-PCR (Table 2) *(16)*. The complete envelope nucleotide sequences of this isolate (accession nos. KP893717) and the two serum-derived DENV3 isolates from different patients with classical dengue fever in Hai Phong, Vietnam (accession nos. KP893718 and KP893719) were determined and deposited in Genbank. Nucleotides sequences were aligned by using MAFFT version 7.215 *(17)*. The substitution models were selected by jmodeltest-

2.1.7 *(18)* and GTR+I+G was used as the model. Phylogenetic tree was constructed by FigTree software, version 1.4.0. The envelope sequence of the three strains did not show any difference in nucleotide and amino acid substitutions. However, the ongoing full genome analysis showed Leu-3029-Phe and Thr-4077-Ile mutations in non-structural regions 1 and 2A (NS1& NS2A) sequences respectively in CSF derived DENV3 strain (data not shown).

Phylogenetic analysis showed the close relationship between this CSF-derived DENV3 and the two serum-derived DENV3 strains and all of them were isolated from different patients in Hai phong, Vietnam in 2013 and were determined to belong to genotype III (Figure 1). Previously, genotype II had been circulating in Vietnam (Figure 1). DENV3 genotype III was reported to be continuously circulating in the Indian subcontinent since the 1960s *(19)*. Since 2005, this genotype III of DENV3 was increasingly found in Bhutan, Thailand, Laos, Cambodia, Pakistan, China, Senegal and Côte d'Ivoire *(19-22)* (Figure 1). To our knowledge, this is the first report describing the emergence of DENV3 genotype III in Vietnam in 2013.

In conclusion, dengue encephalitis is a rarely reported infection, however, its detection tends to increase the threat to global human health. Despite the unusual manifestations and challenges in diagnosis, DE should be investigated further in all patients with encephalitis regardless of the absence of classical dengue features.

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Competing interests:

The authors have declared that no competing interests exist.

Ethical approval:

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

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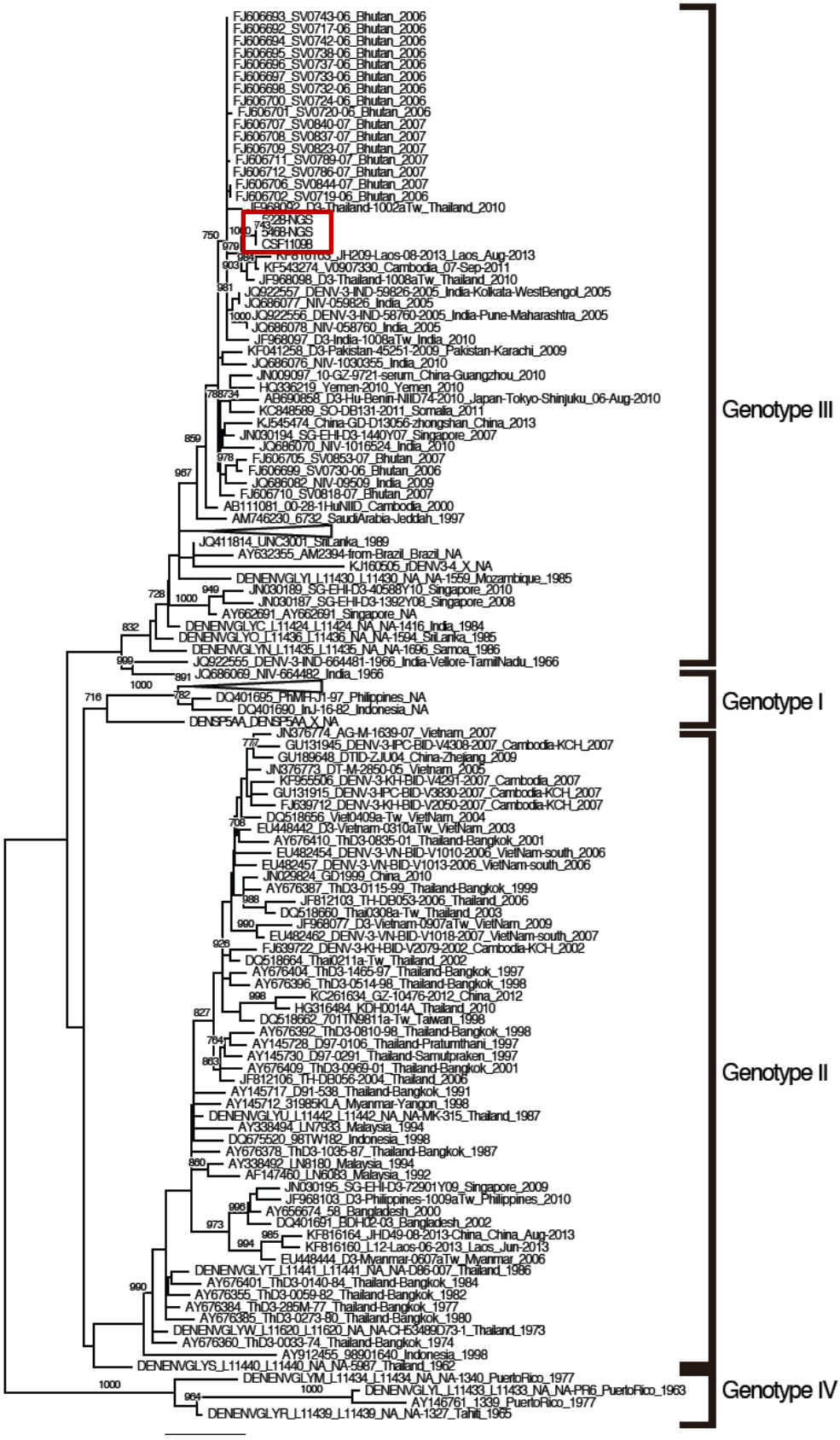
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DENENVGLYL_L11433_L11433_NA_NA-PR6_PuertoRico_1963
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DENENVGLYR_L11439_L11439_NA_NA-1327_Tahiti_1965
Genotype III
Genotype I
Genotype II
Genotype IV
0.04

Figure 1: ML-phylogenetic tree based on the envelope gene sequence of DENV3 isolates. The envelope sequence of DENV3 (CSF11098) isolated from the CSF of our case-patient in Hai Phong,Vietnam in 2013 was compared with those of DENV3 (5228 NGS and 5468 NGS) isolated from serum samples of other patients in the same place and in the same year. The strain names of these three DENV3 isolates were enclosed in red square. This fragment of CSF derived DENV3 was also compared with others homologous sequences of DENV3 in GenBank database from different geographical regions. Bootstrap values over 800 > of 1000 repeats are shown at the nodes. Labels of strains conform to the following format: (GenBank accession nos)_(Strain name)_(Country-region)_(Year of isolation).

Table 1: Summary of laboratory analysis during admission

Hospital	**Viet tiep's hospital**	**Referral hospital**		
Day of onset	1	3	6	9
Hemogram				
– *WBC ($x10^3$/ul)*	5.7	2.72	2.09	5.28
– *Hb (g/dl)*	11.4	12.5	13	12.9
– *Hct (%)*	34.5	36.7	39	38.3
– *PLT ($x10^3$/ul)*	274	220	128	201
CRP (mg/l)	12	0.79	ND	ND
AST (U/l)	40	55	ND	ND
ALT (U/l)	28	50	ND	ND
Dengue serology test				
– *Anti – dengue IgM antibody*	ND	ND	Pos	ND
– *Anti – dengue IgG antibody*	ND	ND	Pos	ND
CSF analysis				
– *WBC/mm^3*	$10^{(*)}$	8	ND	ND
– *Protein (g/l)*	ND	0.46	ND	ND
– *Glucose (mmol/l)*	ND	5.2	ND	ND
– *Gram staining*	ND	Neg	ND	ND
– *Bacterial isolation*	ND	Neg	ND	ND

WBC: white blood cells; Hb: Hemoglobin; Hct: Hematocrit; PLT: platelet; CRP: C-reactive protein (normal range: <0.5mg/dl); AST: aspartate transaminase (normal range: <37 U/l); ALT: alanine transaminase (normal range: <41 U/l); CSF: cerebral spinal fluid; Pos: positive; Neg:

negative; ND: not done

(*): 60% polymorphs and 40% lymphocytes

Table 2: Primers use for RT-PCR and amplification of DENV3 envelope gene.

Primer name	**Reaction**	**sense**	**Sequence (5'-3')**
Random primer	RT-PCR	-	
VNR primer	RT-PCR	-	AGAACCTGTTGATTCAACAGCACCATTCCA
DENV-consensus	PCR	F	TCAATATGCTGAAACGCGCGAGAAACCG
DENV-consensus	PCR	R	TTGCACCAACAGTCAATGTCTTCAGGTTC
DENV-3-specific	PCR	F	GTGCTTACACAGCCCTATTT
DENV-3-specific	PCR	R	TCCATTCTCCCAAGCGCCTG

RT-PCR: reverse transcriptase-polymerase chain reaction; F: forward; R: reverse; DENV: dengue virus.