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Japanese encephalitis virus genotype III from mosquitoes in Tarlac, Philippines

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ABSTRACT

Objectives: The aim of this study was to investigate the presence of Japanese encephalitis virus (JEV) in a rice-farming community in the Philippines and to determine its implications regarding the epidemiology of viral encephalitides in the Asia-Pacific Region.

Methods: Mosquitoes were collected monthly from animal-baited traps close to flooded rice fields in two barangays (villages) in the Municipality of San Jose, Tarlac Province in Luzon, from May 2009 to July 2010. Virus was detected by nested reverse transcription PCR. Phylogenetic analysis of the amplified virus envelope gene was done using the maximum-likelihood method.

Results: A total of 28 700 known vector mosquitoes were collected, namely *Culex vishnui*, *Culex fuscocephala*, *Culex tritaeniorhynchus*, and *Culex gelidus*. JEV genotype III was detected in *C. tritaeniorhynchus*, belonging to the same genotype but form a different clade from those reported in the 1980s and in 2020 in this country.

Conclusions: Japanese encephalitis is associated with rice cultivation and the presence of infected mosquitoes in Tarlac, Philippines. It remains to be seen whether the observed genetic shift of genotype III to genotype I in Asia will in time have an impact on the epidemiology of Japanese encephalitis in the Philippines. For long-term disease control, regular surveillance and Japanese encephalitis immunization in children and travelers in high risk areas are recommended.

1. Introduction

Japanese encephalitis (JE) is a potentially fatal mosquito-borne infectious disease that affects three billion people in 27 countries in the Asia-Pacific region (Erlanger et al., 2009; Wang and Liang, 2015). About 40 000 out of 69 000 clinical cases are estimated to occur annually in the Western Pacific region, which includes the Philippines (Lopez et al., 2015). The global incidence rate is 1.8/100 000 population, with approximately 13 600–20 400 deaths (Campbell et al., 2011; WHO, 2015b). Individuals of any age are at risk, but especially children 2–15 years old. The disease causes inflammation of the

brain with headache, fever, weakness, changes in sensorium, paralysis, and seizures, especially in children. There is no specific antiviral treatment, and supportive or rehabilitative care is provided to alleviate the neurological sequelae in 30–50% of survivors (WHO, 2015a). To prevent infection, anti-Japanese encephalitis virus (JEV) vaccination is recommended for children and travelers in high risk areas (MMWR, 2013).

Several formulations of JE vaccines are currently in use in countries with a routine national immunization program in children (WHO, 2006; Hills et al., 2019). These include a live-attenuated vaccine, Vero cell-derived inactivated vaccine, and live chimeric vaccine with yellow fever

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17D as the backbone. A cost-effectiveness analysis comparing alternative delivery strategies by Vodicka et al. (2020) supports the use of CD-JEV for JE control in the Philippines. CD-JEV is a live-attenuated vaccine against JE that was developed in 1980 by Chengdu Institute of Biological Products in China. This vaccine is recommended by the World Health Organization (WHO), and CD-JEV has been used for 30 years in endemic countries. It is the least expensive vaccine on the market and is given via subcutaneous injection in a single dose of 0.5 ml to adults and children 8 months of age and older.

Two epidemiological patterns have been observed for JE (Vaughn and Hoke, 1992; Solomon et al., 2000). In temperate zones, such as the northern part of the Korean peninsula, Japan, China, Nepal, and northern India, epidemics occur in the summer. In the tropical areas of southern Vietnam, southern Thailand, Indonesia, Malaysia, the Philippines, and Sri Lanka, cases are more sporadic and are generally seen during the rainy season (Wang et al., 2009; Lee et al., 2012; Saito et al., 2015; Garjito et al., 2018; Kumar et al., 2018; Yap et al., 2019; Zheng et al., 2012). In the Philippines, JE occurs following an increase in mosquito populations with the onset of rains or the irrigation of rice fields during the planting season (Hayes et al., 1986; Shultz and Hayes, 1993; Keiser et al., 2005).

In nature, the enzootic cycle of JE is maintained between domestic and wild animals and mosquitoes. Humans are considered to be dead-end hosts, since they do not develop sufficient viremia to infect mosquitoes (Pearce et al., 2018). Mosquitoes of the genus *Culex* are known vectors, foremost among which is *Culex tritaeniorhynchus*. An ecological niche model estimated that 46% of the land area of the Philippines had a >25% probability of the presence of the vector, *C. tritaeniorhynchus* (Miller et al., 2012). This is a concern in rice-growing areas, where large populations live in close proximity to mosquito habitats. JEV nonetheless utilizes a wide range of culicine and anopheline vector species, sharing some of these with other mosquito-borne diseases such as dengue, Zika, chikungunya, filariasis, and malaria (Ritchie et al., 1997; Okuno et al., 1971; Chunsuttiwat, 1989; Thenmozshi et al., 2006; Vythilingam et al., 2002). Shultz and Hayes (1993) identified 44 species of mosquitoes under eight genera in an endemic area in Luzon, with *Culex vishnui* being the most abundant. Since the mosquitoes that carry JEV breed in flooded rice fields and pools of water, most human infections occur in rural, agricultural areas in Asia, although cases have been reported from peri-urban situations, such as in Phnom Penh, Cambodia (Cappelle et al., 2016).

Hogs and wading ardeid birds serve as amplifying and reservoir hosts, respectively, and their involvement as such was demonstrated as early as 1938 by Tsai and colleagues. Confirmed JE cases have been found among people living in the vicinity of rice paddies and pig-raising farms. (Natividad et al., 2006; Lopez et al., 2015). Elsewhere virus and/or antibodies have been detected in domestic and wild animals, including swine, bats, horses, donkeys, cattle, sheep, dogs, cats, reptiles, amphibians, and insects (Campos et al., 1966; Chan, 1999; Wang and Liang, 2015; Ohno et al., 2009; Yap et al., 2019). Migratory birds may play a role in spreading the virus overseas or in non-contiguous areas (Nitapattana et al., 2008). There is evidence of mosquitoes being carried by the wind from Papua New Guinea to Northern Australia (Ritchie et al., 1997; Ritchie and Rochester, 2001).

JEV is a single-stranded, positive-sense, enveloped, icosahedral RNA virus belonging to the family *Flaviviridae*, which includes dengue, yellow fever, West Nile encephalitis, and Zika viruses (Solomon, 2004). From phylogenetic analysis of JEV strains, there are four genotypes identified from the precursor membrane envelope protein (prM gene) and five genotypes from the E gene and full-length genome (Wang and Liang, 2015). These genotypes (GI–GV) circulate singly or in combination among countries and have been observed to cause disease outbreaks periodically. GIII was the dominant genotype in Asia from 1935 to the 1990s (Solomon et al., 2003). Subsequently, endemic countries in Asia have seen a genetic shift in circulation from GIII to GI (Schuh et al., 2014; Do et al., 2015; Han et al., 2015). Schuh et al. attributed GI re-

placement of GIII to an increase in multiplicative ability and higher infectivity titers for GI compared to GIII isolates, as observed experimentally *in vitro* in mosquito cells. The higher reproductive kinetics of the GI strain translates to a shorter extrinsic incubation period in the mosquito and thus an increased transmission potential (Han et al., 2014).

The history of JE dates back to the 19th century, with the first clinical case seen in 1871 in Japan, and post mortem identification of the virus in 1935, recovered earlier from human brain tissue (Erlanger, 2009). The epidemiology of JE has been the subject of comprehensive reviews in recent years. Lopez et al. (2015) established the chronology of JE in the Philippines based on information gathered from serological surveys, animal studies, case reports, and geographic distribution and surveillance data from 2011 to 2014. Anti-JEV antibodies were detected in horses in the Philippines as early as 1943, and the first serologically confirmed case was recorded in 1956, in an American soldier stationed at a military base in the country (Hammon et al., 1958). In a seroprevalence survey by Hammon et al., JEV was identified among indigenous children residing near the military base in Pampanga. The first outbreak of JE occurred in Nueva Ecija (Ksiazek et al., 1980). Both provinces are located in Central Luzon or Administrative Region 3. The first known isolation of JEV from mosquitoes was from *C. tritaeniorhynchus* and *C. vishnui* collected in Tagudin, Ilocos Sur Province in Northern Luzon, Administrative Region 1 (Trosper et al., 1980). JEV was isolated from *C. tritaeniorhynchus*, *Culex bitaeniorhynchus*, and *Anopheles annularis* mosquitoes in Nueva Ecija by Ksiazek et al. in 1980. Shultz and Hayes detected anti-JEV antibodies in pigs from Nueva Ecija in 1993.

In 2003, seven patients were diagnosed with JE in the town of Tarlac, about 100 km north of the city of Manila. Subsequently the extent of JEV infection in mosquitoes and swine in the Municipality of San Jose in Tarlac Province was investigated from 2009 to 2011. To facilitate the confirmation of cases, the Research Institute for Tropical Medicine was designated as the national referral center of the WHO JE laboratory network in the Western Pacific Region in 2009. JE has been documented in Metro Manila and in 32 provinces, while 68 out of 81 provinces and major cities have reported suspected JE cases with at least one case each per 17 regions (Lopez et al., 2015).

The aim of this study was to investigate the presence of Japanese encephalitis virus (JEV) in a rice-farming community in the Philippines and to determine its implications regarding the epidemiology of viral encephalitides in the Asia-Pacific Region.

2. Methods

2.1. Collection and identification of mosquitoes

Mosquitoes were collected monthly from animal-baited traps in Barangay Moriones (15.45° N, 120.47° E, population 3300 as of 2010) and Barangay Lubigan (15.44° N, 129.45° E, population 1356 as of 2010) in the Municipality of San Jose, Tarlac Province in Central Luzon (<http://www.philatlas.com/luzon/r03/tarlac/san-jose.html>), from May 2009 to July 2010 (Figure 1). The trap consisted of a white, rectangular nylon mesh measuring 4 m × 3 m × 3 m with a 1 m² retractable flap covering an opening situated on one side of the net (Figure 2). The net was installed in a flat area and tethered to posts or nearby trees. With the native buffalo, *Bubalus bubalis* inside, the entrance was left open from 6:00 pm to 4:00 am, after which the animal was removed and the cover was lowered to prevent trapped mosquitoes from escaping. The mosquitoes were collected individually with a mouth aspirator, and 50–100 were placed in a plastic screened cup with a 1-cm hole for transferring mosquitoes with the aspirator. An electric-operated vacuum apparatus was also used to collect mosquitoes from the trap. While in the field, the mosquitoes were kept alive with a cotton plug soaked in sugar solution. The mosquitoes in each cup were then immobilized, sorted by genus, and identified to the species level under a stereoscope with the

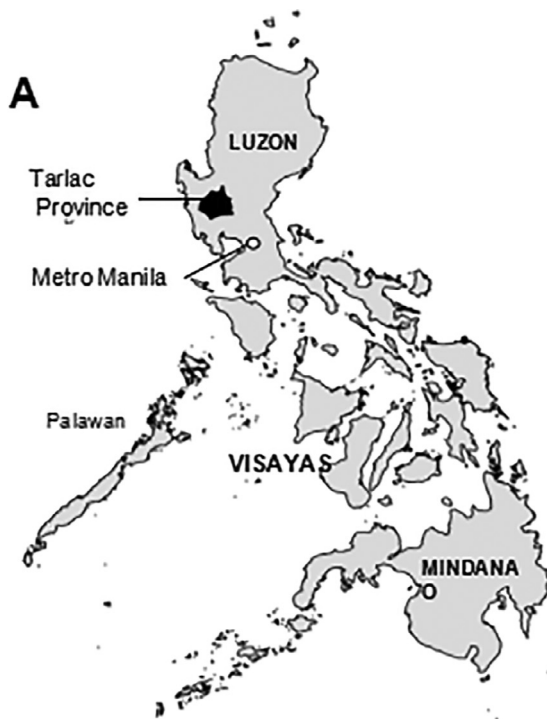


Figure 1. Location of the study site in the Municipality of San Jose, Tarlac, Philippines. (A) Tarlac Province (black) in Central Luzon, Philippines. (B) Barangays Lubigan and Moriones (light gray) in the center of Tarlac Province. Boundaries of the municipalities in Tarlac Province and the Municipality of San Jose (dark gray and light gray) are shown.



Figure 2. Animal-baited trap for the collection of mosquitoes.

use of taxonomic keys (Rattanaarithikul et al., 2005). The place and date of collection were recorded. The mosquitoes were kept frozen prior to homogenization.

2.2. Mosquito homogenization

Mosquitoes classified by species were pooled into tubes of 50–100 mosquitoes each. The JEV detection manual published by the National Institute for Infectious Diseases recommend using 1–100 mosquitoes per pool (NIID, 2003). The pooled mosquitoes were homogenized in 5 ml sterile plastic tubes containing two glass beads (3.2 mm diameter; Wakenyaku, Kyoto, Japan) and 2 ml of Eagles-minimum essential medium (MEM) cell culture medium containing 2% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acid, penicillin, streptomycin, and amphotericin B (Fungizone; Gibco, Invitrogen, NY, USA) as per the procedure of Hoshino et al. (2009). The samples were centrifuged at 3000 rpm for 15 min at 4°C, and the supernatants were then collected and stored at –80°C until use.

2.3. Detection of JEV by RT-PCR

Viral ribonucleic acid (RNA) was extracted from the mosquitoes using a Purelink Viral RNA/DNA mini kit. The amount of viral RNA after elution was not measured. The viral RNA was reverse-transcribed to complementary DNA (cDNA) using Moloney murine leukemia virus (M-MLV) reverse transcriptase random primers (Invitrogen, Carlsbad, CA, USA). A nested PCR was performed to amplify the envelope gene of JEV based on the protocol published by the National Institute for Infectious Diseases with some modifications (NIID, 2003). The reaction mixture contained 0.4 µl of 10 µM forward primer, 0.4 µM reverse primer, 1 µl of template cDNA, 5 µl of 5X Ex Taq buffer, 0.8 µl of 2.5 nM dNTP mix, and 1 µl of Ex Taq. RNase-free water was added to a total volume of 10 µl. The primer set for the first RT-PCR was JEBK-S: 5'-ATG GAA CCC CCC TTC-3' and JEER: 5'-AGC AGG CAC ATT GGT CGT TA-3.

The primer set for the nested PCR was JEBK inner-S: 5'-AGC ACA CCT CCT GTG GCT AA-3' and JEER inner-C: 5'-AGC ACA CCT GTG GCT AA-3 [Au?7]. PCR cycling conditions were as follows: 1 cycle at 94°C for 5 min; 40 cycles at 94°C for 30 s, followed by 50°C for 30 s, 72°C for 30 s and a final extension at 72°C for 10 min. The PCR products were separated by gel electrophoresis and visualized under UV light. The specific size of the PCR product after nested PCR was 326 base pairs.

2.4. Sequencing and phylogenetic analysis

A SUPREC PCR Purification Kit (Takara Bio Inc., Shiga, Japan) was used to purify the PCR products prior to nucleotide sequencing. Cycle sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using the aforementioned primers. Sequencing reactions were purified using a BigDye XTerminator Purification Kit (Applied Biosystems) followed by loading into a 3130 or 3730xl genetic DNA analyzer. The accession numbers of the nucleotide sequences of the JEV obtained in this study are **LC602822** and **LC602823**.

Multiple sequence alignments and phylogenetic relationships were inferred using the maximum-likelihood method of the Molecular Evolutionary Genetics Analysis version 5 algorithm MEGA 5

Table 1
Monthly collections of mosquitoes from May 2009 to July 2010

Month-year	Rainfall (mm)	Vector <i>Culex</i> species				Total
		<i>C. vishnui</i>	<i>C. fuscocephala</i>	<i>C. tritaeniorhynchus</i>	<i>C. gelidus</i>	
May-09	506.7	673	290	389	110	1462
Jun-09	621.2	1561	587	531	33	2712
Jul-09	343.5	825	123	240	11	1199
Aug-09	318.6	320	325	47	56	748
Sep-09	547.6	-	-	-	-	-
Oct-09	219.1	417	110	200	74	801
Nov-09	40.1	740	1028	818 ^a	49	2635
Dec-09	5.2	770	801	287	342	2200
Jan-10	18.8	1001	527	253	114	1895
Feb-10	0	732	198	56	15	1001
Mar-10	3.6	143	83	92	5	323
Apr-10	36.8	40	12	19	1	72
May-10	27.6	-	-	-	-	-
Jun-10	214	778	132	718	17	1645
Jul-10	265	4561	5831	1434 ^b	181	12 007
Total	3167.8	12 561	10 047	5084	1008	28 700

^a positive for JEV in Nov. 2009.

^b positive for JEV in July 2010.

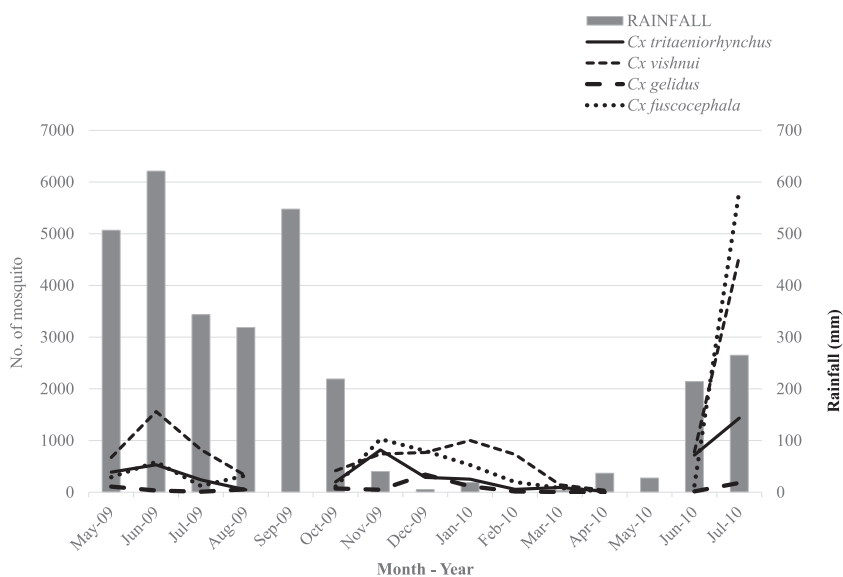


Figure 3. Vector mosquito populations and monthly rainfall from May 2009 to July 2010. (Rainfall data from the Philippine Atmospheric, Geophysical and Astronomical Services Administration (PAGASA), Climatology and Agrometeorology Division.)

(<http://www.megasoftware.net>) with bootstrap probability calculated from 1000 replicates. All reference sequences used for comparative analyses were obtained from the GenBank genetic sequence database with accession numbers.

3. Results

Mosquitoes were found to be present in the study area from May 2009 to July 2010. Four known vector species were identified. In decreasing order of abundance, these were *C. vishnui*, *Culex fuscocephala*, *C. tritaeniorhynchus*, and *Culex gelidus* (Table 1). Populations of *C. tritaeniorhynchus* and *C. fuscocephala* peaked in November 2009. Rainfall was low from December 2009 to March 2010, while residual pools of water sustained mosquito breeding habitats. In September 2009 the devastating effects of Typhoon Ketsana, known in the Philippines as Tropical Storm Ondoy, interrupted entomological activity, hence mosquito collection was not done (Figure 3). No collection was done in May 2010.

Collection was resumed in June and July 2010 when rainfall was starting to peak, during which two animal-baited traps were added and vacuum aspirators were used to collect mosquitoes. *C. vishnui* and *C.*

fuscocephala continued to outnumber *C. tritaeniorhynchus* and *C. gelidus*. Entomological activities were discontinued after July 2010.

A total of 333 pools of mosquitoes consisting of 46 pools of *C. fuscocephala*, 23 pools of *C. gelidus*, 171 pools of *C. tritaeniorhynchus*, and 93 pools of *C. vishnui* were examined by nested RT-PCR test. JEV was detected in one pool of *C. tritaeniorhynchus* collected in November 2009 from Barangay Moriones and one pool of *C. tritaeniorhynchus* collected in July 2010 from Barangay Lubigan (Table 2).

Phylogenetic analysis revealed that these two strains belonged to GIII, the same genotype but different clade from Philippine strains collected from pigs in Mindanao by Kuwata et al. in 2016–2018 and from pigs in 1984–1986 (Kuwata et al., 2020). The JEV strains reported in this study were the closest to strains from South China, with 96.9% similarity, while the similarity with previous Philippine strains and 2016–2018 Mindanao strains were 94.5–96.5% and 92.8–94%, respectively (Figure 4).

4. Discussion

The significance of this study lies in the fact that Tarlac is one of seven provinces of Central Luzon, a major rice-producing area in

Table 2
JEV was detected in two pools of *Culex tritaeniorhynchus* collected one each for the two Barangays of Lubigan and Moriones

Vector species	Municipality of San Jose, Tarlac Province				Total	
	Barangay Moriones ^a		Barangay Lubigan ^b		No.	%
	No.	%	No.	%		
<i>Culex vishnui</i>	8704	44	3857	44.2	12 561	43.8
<i>Culex fuscocephala</i>	7247	36	2800	32.1	10 047	35
<i>Culex tritaeniorhynchus</i>	3397	17	1687	19.3	5084	17.7
<i>Culex gelidus</i>	638	3	370	4.3	1008	0.3.5
All species	19 986	100	8716	99.9	28 700	100

^a positive for JEV in Nov. 2009.

^b positive for JEV in July 2010.

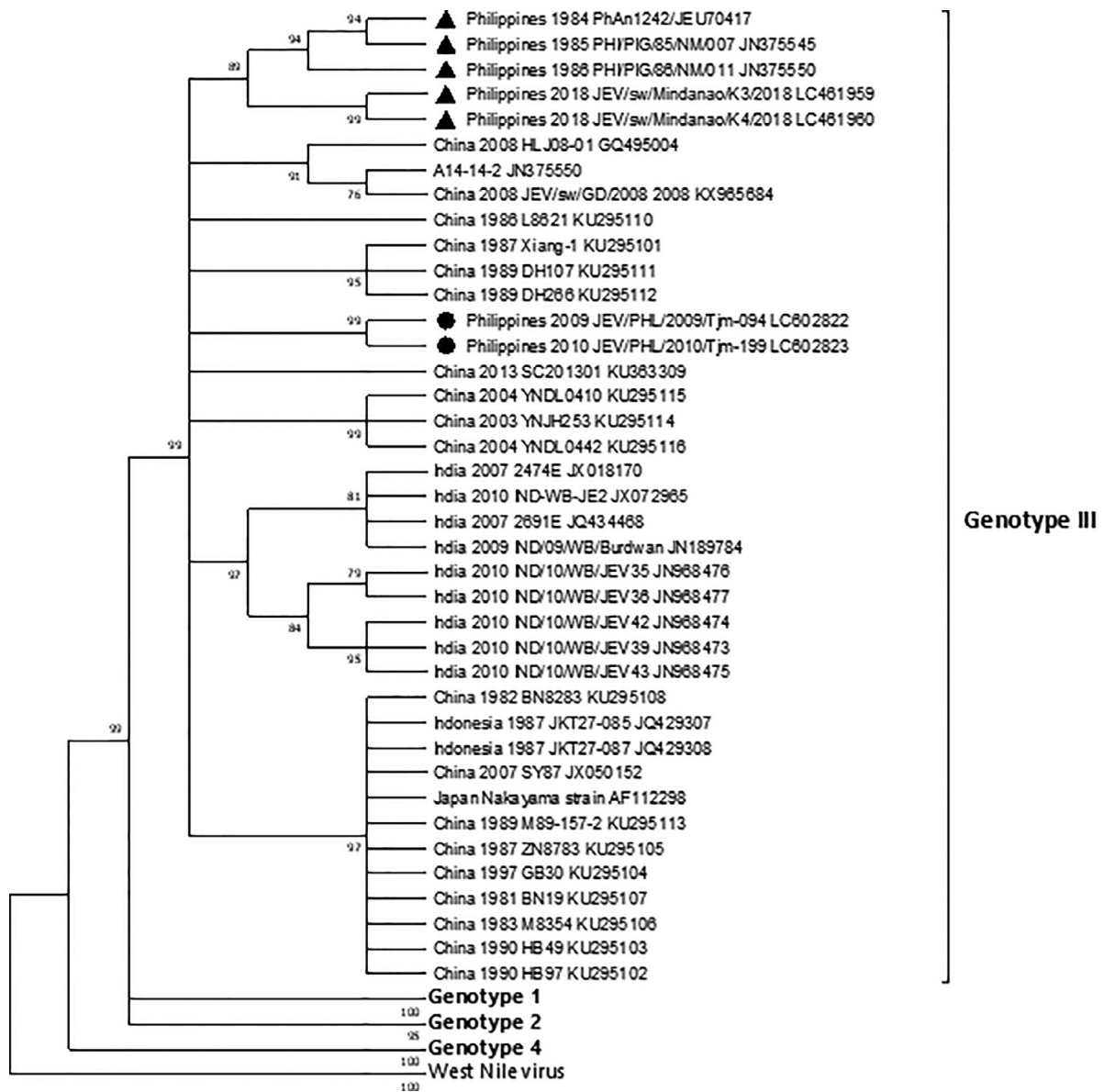


Figure 4. Phylogenetic tree of the partial envelope gene of Japanese encephalitis virus. The phylogenetic tree was constructed using the partial envelope gene (333 nt) by maximum-likelihood method. The bootstrap value was calculated from 1000 replicates, and replicates reproduced in less than 70% bootstrap were collapsed [Au?3]. Black circles indicate the samples detected in this study and black triangles indicate the Philippine strains reported in the 1980s and 2018.

the country. The study site, San Jose, is a third class municipality (population 36 253 as of 2010) with an average annual income of 5–10 million pesos (<https://www.philatlas.com/luzon/r03/tarlac/san-jose.html>). Domestic hog raising is a common practice among farmers here in San Jose which is the largest municipality of the province with 13 barangays or villages. In a parallel study conducted in 2010–2011, virus-infected and seropositive piglets were found in five barangays in San Jose (unpublished data). Rice farming coincides with the rainy season from May to November. Egrets and herons come foraging for food in the rice fields. In addition, Candaba Swamp, a 32 000 hectare wetland in Pampanga, within 100 km from Tarlac, is on the route of migratory birds (de Vera-Ruiz, 2019). Migratory birds could serve to spread JE along their path of migration, thus all of the conditions for JE transmission exist in San Jose.

The strains circulating in Tarlac Province belonged to a different clade from earlier Philippine strains and more recent Mindanao strains, and have been maintained for a long time in the country. It appears that the introduction of JEV from other countries to the Philippines might be limited. The ancestral virus with all five genotypes is presumed to have originated from the Indonesian-Malaysia archipelago and dispersed from there as far north as Japan and South Korea, to India and Pakistan eastward and west towards Australia (Reuben and Gajanana, 1997; Solomon et al., 2003).

The five genotypes became dispersed among six geographical regions according to the prevailing strains, as follows: (1) Indonesia (excluding New Guinea) and Malaysia, GI–V; (2) Australia and New Guinea, GI and GII; (3) Taiwan and the Philippines, GII and GIII; (4) Thailand, Cambodia, and Vietnam, GI, GII, and GIII; (5) Japan, Korea, and China, GI and GIII; and (6) India, Sri Lanka, and Nepal, GIII. GI consists of two clades, GIa and GIb, with the latter being associated with the displacement of GIII as the dominant JEV genotype throughout Asia in the 1990s (Solomon et al., 2003). Phylogeographic analysis indicated that GIa diverged in Thailand or Cambodia and has remained confined to tropical Asia, whereas GIb diverged in Vietnam and then dispersed northwards to China, where it was subsequently dispersed to Japan, Korea, and Taiwan (Schuh et al., 2014). On the other hand, the JEV isolate from Thailand reported by Kuwata et al. in 2017 was GIb and clustered with JEV isolates obtained during 1985–2005 (Nitapattana et al., 2008). Before 2008, all of the JEV found in Taiwan belonged to GIII. GI was first identified in northern Taiwan in 2008. Following this, the proportion of GI isolates in Taiwan increased rapidly from 2009 to 2010 (Su et al., 2014). GI became the predominant JEV genotype circulating in Taiwan. Since 2011, almost all of the JEV isolates obtained in Taiwan have belonged to GI, with the exception of two GIII strains found in Kuantu Nature Park in Taipei City in 2012. Su et al. (2014) reported that GIII strains isolated in Taiwan between 2005 and 2012 were divided into two clusters (clusters 1 and 2). Cluster 2 strains of GIII are closely related to viruses from China, Japan, Indonesia, and the Philippines. Kuwata et al. (2020) claimed that GIII is still active in many parts of Asia. Meanwhile GIII to GI genetic shift remains to be studied in the Philippines.

In conclusion, the conditions for the acquisition and spread of Japanese encephalitis are associated with rice-farming and the presence of infected mosquitoes and swine in Tarlac and adjoining provinces in Central Luzon. This situation is typical in many such communities where rice cultivation and backyard hog-raising are common household enterprises. Although the occurrence is sporadic, affecting mostly children below 15 years of age, periodic surveillance and laboratory confirmation of cases are needed to monitor virus activity, with the inclusion of anti-JEV vaccination in the national immunization program. Access of mosquitoes to both human and animal amplifying hosts can be prevented by relocating piggens away from residences and rice fields. The use of mosquito repellents and insecticide-treated bed nets could protect against night-biting vector species. It remains to be seen whether the GIII to GI genetic shift will have an impact on the epidemiology of Japanese encephalitis in the Philippines.

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Ethical review

The study was granted ethical clearance by the Institutional Review Board of the Research Institute for Tropical Medicine, Department of Health, Philippines, on August 10, 2009.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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