Molecular Epidemiology of Japanese Encephalitis Virus in Mosquitoes in Taiwan during 2005–2012

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Abstract

Japanese encephalitis (JE) is a mosquito-borne zoonotic disease caused by the Japanese encephalitis virus (JEV). Pigs and water birds are the main amplifying and maintenance hosts of the virus. In this study, we conducted a JEV survey in mosquitoes captured in pig farms and water bird wetland habitats in Taiwan during 2005 to 2012. A total of 102,633 mosquitoes were collected. Culex tritaeniorynchus was the most common mosquito species found in the pig farms and wetlands. Among the 26 mosquito species collected, 11 tested positive for JEV by RT-PCR, including Cx. tritaeniorynchus, Cx. annulus, Aedes sinensis, Armigeres subalbatus, and Cx. fuscoccephala. Among those testing positive, Cx. tritaeniorynchus was the predominant vector species for the transmission of JEV genotypes I and III in Taiwan. The JEV infection rate was significantly higher in the mosquitoes from the pig farms than those from the wetlands. A phylogenetic analysis of the JEV envelope gene sequences isolated from the captured mosquitoes demonstrated that the predominant JEV genotype has shifted from genotype III to genotype I (GI), providing evidence for transmission cycle maintenance and multiple introductions of the GI strains in Taiwan during 2008 to 2012. This study demonstrates the intense JEV transmission activity in Taiwan, highlights the importance of JE vaccination for controlling the epidemic, and provides valuable information for the assessment of the vaccine’s efficacy.

Introduction

Japanese encephalitis is a vector-borne zoonotic disease transmitted by the bite of a JEV-infected mosquito. Although JE is a vaccine preventable disease, JEV infections are still the leading cause of viral encephalitis in Asia [1], [2]. It is estimated that 67,900 JE cases occur annually in JE endemic countries, with an incidence rate of 1.5 cases per 100,000 individuals [3]. Symptoms of JE include fever, chills, headache, myalgia, weakness, mental disturbances and neurologic symptoms. The mortality rate can reach as high as 30%, and approximately 30–50% of survivors suffer severe neurological damage [4], [5].

The JEV belongs to the genus Flavivirus of the family Flaviviridae and is a single-stranded positive-sense RNA virus. The viral genome is approximately 11 kb in length and encodes three structural proteins [capsid (C), premembrane (prM), and envelope (E)], followed by 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) [6], [7]. According to phylogenetic analysis of the E protein gene sequences, the JEV strains can be classified into 5 distinct genotypes (genotypes I–V) [8], [9]. Recent studies suggested that the epidemic/dominant genotype of JEV has gradually shifted from genotype III (GIII) to genotype I (GI) in Southeast and East Asian countries in the last two decades [10–14].

Japanese encephalitis is an endemic disease in Taiwan and has been designated as a notifiable infectious disease since 1955. The highest incidence rate of confirmed JE cases (2.05 per 100,000) was recorded in 1967. After the mass JE vaccination program was implemented in 1968, the incidence rate of confirmed JE cases declined significantly [15–17]. From 1998 to 2011, the annual number of confirmed JE cases ranged from 13 to 37. In 2012, 32 JE cases were confirmed, which is equivalent to an incidence rate of 0.13 per 100,000 individuals. From 1998 to 2012, the epidemic peak months were June and July. Confirmed cases occurred sporadically throughout Taiwan. Most individuals diagnosed with JE lived near rice paddy fields or pig farms [18].

We previously reported the molecular epidemiology of JEV in Taiwan [19]. The study demonstrated that all known JEV isolates collected before 2008 belonged to GII. Genotype I JEV strains that were first found in northern Taiwan in 2008. In the present study, we monitored the dynamics of the genotype transition and genetic variation of JEV and identified the mosquito species potentially involved in the transmission of JEV in Taiwan during 2005–2012.

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Supporting Information files except for the nucleotide sequences of JEV strains (obtained in this study) which are available from Genbank under the accession numbers: KF667277-KF667327.

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Author Summary

Japanese encephalitis (JE) is a vector-borne zoonotic disease transmitted by the bite of a Japanese encephalitis virus (JEV) infected mosquito. Japanese encephalitis is an endemic disease in Taiwan. Before 2008, all known JEV isolates collected in Taiwan belonged to Genotype III of JEV. Genotype I JEV strains were first found in northern Taiwan in 2008. In this study, we conducted a survey of JEV in mosquitoes during 2005–2012. A total of 102,633 mosquitoes were collected from pig farms and wetlands. Among the 26 mosquito species collected, 11 tested JEV positive by RT-PCR, including Cx. tritaeniorhynchus, Cx. annulus and An. sinensis. Cx. tritaeniorhynchus was the predominant vector species for transmission of JEV in Taiwan. The JEV infection rate of the mosquitoes captured on the pig farms was significantly higher than the rate of those captured in the wetlands, indicating that pigs played an important role in amplifying JEV. A phylogenetic analysis of the envelope gene sequences of JEV isolated from the mosquitoes demonstrated that the predominant JEV genotype shifted from genotype III to genotype I (GI), providing evidence for multiple introductions and transmission cycle maintenance of GI strains in Taiwan during 2008–2012.

Materials and Methods

Mosquito collections

Mosquitoes were collected on pig farms near rice paddy fields in the northern (Yinge District in New Taipei City and Wuje Township in Yilan County), central (Beitun and Wufeng Districts in Taichung City and Shuili Township in Yunlin County), southern (Xiaying District in Tainan City, Neimen and Alian Districts in Kaohsiung City, Yanyu and Zhutian Townships in Pingtung County), and eastern (Shoufeng and Guangfu Townships in Huaien County) regions of Taiwan and from wetland habitats for water-birds in the northern (Beitun District in Taipei City, and Su’ao Township in Yilan County) and southern (Qigu and Anping Districts in Tainan City) regions from 2005 to 2012 (Figure 1). Mosquito species and infection rates of JEV were calculated using the PooledInfRate software by Biggerstaff (www.cdc.gov/ncidod/dvbid/westnile/software.htm) [24]. The Chi-squared test was used for comparison of the mosquito JEV infection rates of different species and sampling sites.

Viral RNA was extracted from the mosquito suspensions using the QIAamp viral RNA mini kit (Qiagen). Three sets of primers, including flavivirus-specific (FL-F1: 5'-GCCATATGG TA-CATGTTGCTGGGAGC-3', FL-R3: 5'-GTGATTCCTGTGTG- GTCCACWCCGGCTGTGTGATC-3', FL-R4: 5'-GTGATGGCGRGCTGTCACCAGCGRGCAGTGATC-3'), JEV-specific (JEF3F: 5'-CCCTCAGACGTCCTCAGAANAA-3' and JEF3R1: 5'-CTATTCCAGGCGTCAATAATGCTGT-3'), and JEV GII-specific (10F: 5'-CTGGAATGGGCAATCGTG-3' and 5'-TGATCGCTCCCTTCCCTCCC-3') primers, were used for the RT-PCR assay [19], [20]. Real-time RT-PCR was used to screen for JEV in the mosquito pools as previously described [21]. DNA sequences of positive RT-PCR products were determined. JEV positive samples were then subjected to virus isolation.

Virus isolation and genome sequencing

Cell culture techniques using a mosquito C6/36 cell line or plaque assay using the BHK-21 cell line were used for virus isolation as described previously [22]. Viral RNA was extracted from the JEV-infected culture medium using the QIAamp viral RNA mini kit (Qiagen). The primers used for the amplification and sequencing of the complete open reading frame of JEV are listed in Table 1. The RT-PCR reaction was carried out using the Superscript III One-Step RT-PCR system with Platinum Taq High Fidelity (Invitrogen). The RT-PCR reaction was performed under the following parameters: 55°C for 30 min; 94°C for 2 min; 40 cycles of 94°C for 15 sec, 50°C for 30 sec, and 68°C for 1 min; and a prolonged elongation at 68°C for 5 min. RT-PCR products were purified using the Qiagen QIAquick Gel Extraction kit (QiAGEN). Nucleotide sequences were determined using the ABI Prism automated DNA sequencing kit and the ABI Prism 3700 DNA sequencer (Applied Biosystems) according to the manufacturer’s protocols. Overlapping nucleotide sequences were combined and edited using the Lasergene software package (DNASTAR Inc., Madison, WI). Nucleotide sequences of JEV strains were aligned, edited, and analyzed using ClustalW software. The phylogenetic analysis was performed using MEGA 5 [http://www.megasoftware.net/] [23]. The phylogenetic tree was generated using the maximum likelihood method based on the general time-reversible model. The reliability of the analysis was calculated using 1,000 bootstrap replications. The nucleotide sequences of 50 JEV strains isolated from the mosquitoes and a strain isolated from a human were submitted to Genbank with the following accession numbers: KF667277–KF667327.

Infection rates in mosquitoes and statistical analysis

The maximum likelihood estimates (MLEs) of the mosquito JEV infection rate in mosquitoes were calculated using the PooledInfRate software by Biggerstaff (www.cdc.gov/ncidod/dvbid/westnile/software.htm) [24]. The Chi-squared test was used for comparison of the mosquito JEV infection rates of different species and sampling sites.

Results

Mosquito species and infection rates of JEV

Mosquitoes were captured from 16 localities in Taiwan from 2005 to 2012 (Figure 1). A total of 102,633 mosquitoes belonging to the family Culicidae were collected and analyzed. Table 2 shows a summary of the mosquito species, the MLE of the JEV infection rate per 1,000 mosquitoes and the mosquito collection sites (all localities, pig farms, and wetlands). Twenty-six mosquito species from 8 genera of the Culicidae family were identified. The most frequently identified species was Cx. tritaeniorhynchus Giles (36.90%, n = 89,189), followed by Cx. sitiens Wiedemann (6.13%, n = 2,638) and Anopheles sinensis Wiedemann (2.57%, n = 2,638). Of the 2,848 mosquito pools subjected to real-time RT-PCR for the detection of JEV, 499 were positive. The most frequently identified JEV positive mosquito species was Cx. tritaeniorhynchus (468 positive pools), followed by Cx. annulus (9 positive pools) and An.
The MLEs of the JEV infection rates per 1,000 individuals in these three species were 5.85, 8.99, and 2.3, respectively. There were significant differences between JEV infection rates in *Cx. tritaeniorhynchus* and *An. sinensis* at P < 0.05 (P = 0.0357), and in *Cx. annulus* and *An. sinensis* at P < 0.01 (P = 0.0044), but was no significant difference in *Cx. tritaeniorhynchus* and *Cx. annulus* at P < 0.05 (P = 0.0979). *Cx. tritaeniorhynchus* and *An. sinensis* were the most frequently identified mosquito species on the pig farms, whereas *Cx. tritaeniorhynchus* and *Cx. sitiens* were the most frequent in the wetlands. The MLE for the mosquito JEV infection rate was significantly higher on the pig farms (7.5 per 1000) than in the wetlands (1.73 per 1000) (P < 0.01).

Mosquito infection rates of JEV by month

Table 3 shows the MLEs of the JEV infection rate per 1,000 mosquitoes by month and regions. JEV infected mosquitoes first appeared in early May, peaked in June, and then declined in July. The MLEs for the infection rates for May, June, and July were 4.98, 6.72, and 1.46, respectively. May was the peak month in the southern and eastern regions, whereas June was the peak month in the northern and central regions. Figure 2 shows the mosquito JEV infection rates and the numbers of confirmed human JE cases per month during 2005–2012. Spring and summer were the epidemic seasons of JE in Taiwan, and June and July were the peak months for human JE cases.
Table 1. Primers used in RT-PCR and DNA sequencing for Japanese encephalitis virus (JEV).

| Primer name | Sequence 5'-3' | Primer used |
|-------------|----------------|-------------|
| JE-5UTRF    | AGA AGT TTA TCT GTG TGA ACT TCT TGG | PCR, sequencing |
| JE-616R     | CCT CAC ACA TGT AGC CGA CGT CT | Sequencing |
| JE-747R     | TTC GCT TGG AAT GCC TGG TCC G | Sequencing |
| JE-747F     | CGG ACC AGG CAT TCC AAG CGA A | Sequencing |
| JE-1448R    | GGA AGC ATT GAC ACA TGT GCA AAA TT | PCR, sequencing |
| JE-1309F    | AGA ACA ATC CAG CCA GAA AAC ATC | PCR, sequencing |
| JE-1360F    | CGC TGA ATA ATT CCC ATG GTC TTT | Sequencing |
| JE-1839F    | AGG CTG AAA ATG GAC AAA CTG GC | Sequencing |
| JE-1878R    | GGT TGT GCC TTT CAG AGC CAG TTT | Sequencing |
| JE-2515R    | ATC TCT TTT CTT GTG ATG TCA ATG GC | Sequencing |
| JE-2602R    | AGG GAT CTG GCC GTT TCT GG | Sequencing |
| JE-2636R    | GCC TCC CTT GTG GCC TTT GT | PCR, sequencing |
| JE-2340F    | GGG AAT GTC TTG GAT CAC ACA AGG | PCR, sequencing |
| JE-2926F    | TGG AAC AGC ATG CAA ATC GAA GA | Sequencing |
| JE-3032R    | CCT ATG ATC GCT CCA TCA CAG TC | Sequencing |
| JE-3630R    | GGT AGC GAA TGG AAA TCA GAC CTG | PCR, sequencing |
| JE-3467R    | CAA TCT GCC GTG CCA CCT CTG GC | PCR, sequencing |
| JE-4169F    | CCA CTA TAG CTG CCG GAC TAA TGG | Sequencing |
| JE-4324R    | CTG CCA CTA TGA AGG GTA TTG AC | Sequencing |
| JE-4946R    | TGG CAC ACA ACT AGA GGA GCA GC | PCR, sequencing |
| JE-4756F    | GGT TTC TGG TGG ATG TTT ACT GC | PCR, sequencing |
| JE-4946F    | GCA GTA AAC ATC CAG ACA AAA CC | Sequencing |
| JE-5424R    | TGA CAT CAG TCT ATG GTG CAG AGT | Sequencing |
| JE-5424F    | ACT CTG ACC CAT AGA CTG ATG TCA | Sequencing |
| JE-6096R    | CCA TCC CCC ATA ACC AGT GCA AG | PCR, sequencing |
| JE-5946F    | TAA CAT GAT CTT TGC CTC TGT CC | PCR, sequencing |
| JE-6096F    | GGA CAG AGG CAA AGA TCA TGT TA | Sequencing |
| JE-6578F    | GAT GCA GCG AAA GGG TAT AGG GAA | Sequencing |
| JE-6770R    | GTT CCA GGA ACC TCT GCC GCC CA | Sequencing |
| JE-7388R    | TGG ATG GCA AGG AGC ACT CAG | PCR, sequencing |
| JE-7293F    | ACA TCA GTG GGC ACC ATG CCG TC | PCR, sequencing |
| JE-7469F    | CAT AGG GGT AAG GGT GCC AGG GTT | Sequencing |
| JE-7829F    | GAG GAC ATC CGG TTT CCG GAG | Sequencing |
| JE-8030R    | CTC TGC ATG AGC ATC GGT TCT TC | Sequencing |
| JE-8579R    | GCG AAT GGA TCG CAC AGT GTC GAG | PCR, sequencing |
| JE-8403F    | GGA TGC TCA GGG TCT TGG TGC CA | PCR, sequencing |
| JE-8579F    | TGG CAC AAA GAC CCT GAG CAT CC | Sequencing |
| JE-9185R    | GAA CAG AAT CAA TGG AGC ACA GC | Sequencing |
| JE-8926F    | TCT CGG CTC AGC CAA TGG TCT TC | PCR, sequencing |
| JE-9185F    | GAA GAC CAT TGG CTG AGC CCA GA | PCR, sequencing |
| JE-9719R    | TCA AGA GAA GACCAA AGG GAG | Sequencing |
| JE-10266R   | ATG GCC ATG AGC GGA GAC GAC T | PCR, sequencing |
| JE-9489F    | CTG TGT CTT CAA GCC GCT GT | PCR, sequencing |
| JE-9664F    | GTG GCC AAT CTG TCG TCC AGC GG | Sequencing |
| JE-9703F    | GTG GTC ATC CAC TCT CTT TTC CAG | Sequencing |
| JE-10086R   | CCA GAT GTC CTC AGC CTT TCC CAC | Sequencing |
Mosquito infection rates of JEV in different geographical regions

Table 4 shows the MLEs of the mosquito JEV infection rates in different geographical locations. In general, the JEV infection rate was highest in the eastern region (12.32 per 1000) (p<0.01), followed by central (6.02 per 1000) and northern (6.01 per 1000) regions, and lowest in the southern region (1.37 per 1000) (p<0.01).

Genotype shifting of JEV in Taiwan

Table 4 also shows the numbers of GI and GIII JEV positive pools determined by DNA sequencing of RT-PCR products per year. Of the 499 mosquito pools that were JEV positive by realtime RT-PCR, 374 pools were genotyped by sequencing the real time RT-PCR products. The real-time RT-PCR was performed using two sets of primers, one primer set targeting a region of the nonstructural protein 5 (NS5) genes to detect all of the flaviviruses, and the other primer set targeting a region of the 3’ untranslated region (3’UTR) to detect JEV. Both partial NS5 gene sequence (154 bp) and 3’UTR sequence (220 bp) contain sufficient information to differentiate genotypes of JEV. All of the JEV positive pools were genotyped each year, except in 2005 and 2009, only representative RT-PCR positive samples were selected (based on the place and date of collections) for sequencing and genotyping. Figure 3A–3E shows the proportional distribution of the GI and GIII JEV strains in the northern, central, southern, eastern, and all sites of Taiwan between 2005 and 2012. The annual numbers of RT-PCR positive pools for genotype analysis were 99, 42, 2, 42, 47, 56, 33 and 53, respectively, during 2005 to 2012 (Table 4). Before 2008, all the JEV found in Taiwan belonged to GIII. GI was first identified in northern Taiwan in 2008. Since then, the proportion of GI isolates in Taiwan has increased rapidly. From 2009 to 2010, 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 2 GIII strains found in Kuantu Nature Park in Taipei City in 2012. Because GI was the only JEV genotype identified in 2005–2007 and GI was the only genotype found in 2011 (Table 5), to estimate the JEV infection rates according to genotype, we compared the difference between the JEV infection rates in 2005–2007 and 2011. The GIII JEV infection rate per 1,000 mosquitoes in 2005–2007 was 8.86, and the GI JEV infection rate was 6.01 in 2011, there was no significant difference between JEV infection rates in these two groups (P = 0.0514, chi-squared test).

Phylogenetic analysis of JEV isolated from mosquitoes

A total of 148 JEV isolates (147 from Cx. tritaeniorhynchus and one from Cx. annulus) were obtained by virus isolation, and the complete E gene sequences of these isolates were determined. In this study, 50 E gene sequences covered the entire sequence diversity of JEV in Taiwan were selected for phylogenetic analysis. The JEV strains isolated from mosquitoes in Taiwan during 2005–2012 fell into two genotypes (GI and GIII). Figure 4A shows the phylogenetic tree of the E gene sequences of GI, which can be grouped into 2 clusters. Cluster 1 contains the JEV strains isolated from mosquitoes collected throughout the country during 2008–2012, including the first two GI isolates (TPC0806c and YL0806f) identified in Taiwan. The GI virus strains in Cluster 2 were first identified in northern and central Taiwan in 2009; in the following year, these strains were found throughout Taiwan. Cluster 2 also contained a JEV strain isolated from a patient (H10100739) who lived in Kaohsiung City. These strains are most closely related to the viruses from China and Japan. These results indicate that multiple introductions and transmission cycle maintenance of the GI strains occurred during 2008–2012.

Figure 4B shows the phylogenetic tree of GIII JEV. The strains isolated in Taiwan between 2005 and 2012 were divided into 2 clusters (Clusters 1 and 2). The majority of the GIII strains isolated in Taiwan during 2005 to 2011 were classified as Cluster 1. However, in our study, no strains belonging to this lineage were found in 2012. Cluster 2 of GIII included a minor group of JEV strains in Taiwan. Most of the isolates were found in the northern and eastern parts of Taiwan. In 2012, only 2 isolates (TPC1206c-1, TPC1206c-2) belonging to GIII JEV were found in Taiwan. The Cluster 2 strains of GIII are closely related to viruses from China, Japan, Indonesia and the Philippines.

Discussion

In this study, we reported the results of a survey of JEV-infected mosquitoes from pig farms and wetlands in Taiwan during 2005 to 2012. Pig farms near rice paddy fields and wetland habitats for water birds are common in Taiwan, and these places provide suitable environments for the JEV infection cycle [25]. Confirmed JE cases have been identified throughout Taiwan with most of the infected individuals residing near pig farms or rice paddy fields [18]. Cx. tritaeniorhynchus was the predominant mosquito species captured from both the pig farms (92.6%) and the wetlands (76.2%) and was the main species infected with JEV genotypes I and III. Un-baited sweep net sampling method is considered a passive method that can be used to collect a wide variety of mosquito species. Dry ice (CO2)-baited trap can attract host-seeking female mosquitoes, and CO2 appears to be universally

Table 1. Cont.

| Primer name | Sequence 5’-3’ | Primer used |
|-------------|---------------|-------------|
| JE-10230R   | GGT TGC TCT GGA TCG CGT TCC GAT | Sequencing |
| JE-10266F   | ATC GGA AGC CGA TCC AGA GCA ACC | Sequencing |
| JE-10488F   | ACT GGG TAG AGC GTG CTG CCT G | Sequencing |
| JE-10980R   | AGA TCC TGT GTT CTT CCT CAC CAC | PCR, sequencing |
|             |               |             |

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attractive to a variety of mosquito species [26]. Therefore, these two mosquito sampling methods do not seem to have a collection bias towards Cx. tritaeniorhynchus or any of the other mosquito species. These results provide evidence that Cx. tritaeniorhynchus remains a principal vector for the transmission of JEV in Taiwan. Although other Culex mosquitoes, such as Cx. annulus and Cx.

| Table 2. Summary of the mosquito species, the maximum likelihood estimates (MLEs) of the mosquito Japanese encephalitis virus (JEV) infection rate, and the mosquito collection sites (all localities, pig farms, and wetlands). |
|-----------------|-----------------|-----------------|-----------------|
| Species         | All localities  | Pig farms       | Wetlands        |
|                 | MLE (95% CI)²   | No. positive pools/No. pools/No. individuals³ | MLE (95% CI)   | No. positive pools/No. pools/No. individuals³ | MLE (95% CI)   | No. positive pools/No. pools/No. individuals³ |
| Aedes aegypti¹  | 0 (0.00–499.14) | 0/2/3           | 0.00 (0.00–499.14) | 0/2/3 | - | - |
| Ae. albopictus  | 5.38(0.33–25.36) | 1/25/177        | 19.44 (1.30–88.53) | 1/12/46 | 0.00 (0.00–22.75) | 0/13/131 |
| Ae. penganensis²| 0 (0.00–10.46)  | 0/10/283        | - | - | 0.00 (0.00–10.46) | 0/10/283 |
| Ae. vexans      | 12.85 (3.49–34.75) | 3/32/246       | 29.65 (1.91–139.58) | 1/7/32 | 9.75 (1.79–32.33) | 2/25/214 |
| Anopheles ludlowae¹ | 0 (0.00–793.45) | 0/1/1           | 0.00 (0.00–793.45) | 0/1/1 | - | - |
| An. minimus¹    | 52.78 (3.38–230.59) | 1/7/18         | 52.78 (3.38–230.59) | 1/7/18 | - | - |
| An. sinensis    | 2.30 (0.95–4.73) | 6/119/2638      | 2.05 (0.77–4.50) | 5/104/2464 | 5.33 (0.34–25.44) | 1/15/174 |
| An. tessellatus | 3.66 (0.68–11.74) | 2/31/246       | 4.81 (0.33–23.37) | 1/7/180 | 2.75 (0.16–13.24) | 1/24/356 |
| Armigeres subalbatus | 13.49 (3.69–35.78) | 3/30/225 | 17.34 (4.83–45.72) | 3/22/175 | 0.00 (0.00–56.26) | 0/8/50 |
| Coquillettidia crasipes² | 0 (0.00–35.54) | 0/3/47         | - | - | 0.00 (0.00–35.54) | 0/3/47 |
| Culex annulus   | 8.99 (4.67–15.91) | 9/79/991       | 26.29 (13.89–46.52) | 8/46/301 | 1.41 (0.08–6.67) | 1/33/690 |
| Cx. bitaeniorhynchus² | 0 (0.00–37.88) | 0/7/60         | - | - | 0.00 (0.00–37.88) | 0/7/60 |
| Cx. brevipalpis²| 0 (0.00–793.45) | 0/1/1           | - | - | 0.00 (0.00–793.45) | 0/1/1 |
| Cx. fuscanus    | 0 (0.00–450.75) | 0/3/4           | 0.00 (0.00–499.14) | 0/2/3 | 0.00 (0.00–793.45) | 0/1/1 |
| Cx. fuscocephala¹ | 7.77 (2.17–20.82) | 3/19/394      | 7.77 (2.17–20.82) | 3/19/394 | - | - |
| Cx. mimeticus²  | 0 (0.00–793.45) | 0/1/1           | - | - | 0.00 (0.00–793.45) | 0/1/1 |
| Cx. murrelli²   | 0 (0.00–53.12)  | 0/3/39          | - | - | 0.00 (0.00–53.12) | 0/3/39 |
| Cx. nigropunctatus² | 0 (0.00–160.75) | 0/1/9          | - | - | 0.00 (0.00–160.75) | 0/1/9 |
| Cx. quinquefasciatus | 1.51 (0.27–4.95) | 2/74/1333     | 1.64 (0.30–5.38) | 2/67/1226 | 0.00 (0.00–26.51) | 0/7/107 |
| Cx. rubirostris³ | 0 (0.00–42.44)  | 0/8/65          | - | - | 0.00 (0.00–42.44) | 0/8/65 |
| Cx. sitiens³    | 0 (0.00–0.60)   | 0/128/6295      | - | - | 0.00 (0.00–0.60) | 0/128/6295 |
| Cx. tritaeniorhynchus | 5.85 (5.34–6.40) | 468/2242/89189 | 7.68 (6.97–8.45) | 413/1653/61765 | 2.10 (1.60–2.72) | 55/589/27424 |
| Mansonia uniformis | 13.82 (0.79–68.46) | 1/19/75        | 18.14 (1.06–90.71) | 1/12/57 | 0.00 (0.00–146.56) | 0/7/18 |
| Ochlerotatus albotaleratis² | 0 (0.00–793.45) | 0/1/1          | - | - | 0.00 (0.00–793.45) | 0/1/1 |
| Oc. togoi²      | 0 (0.00–793.45) | 0/1/1           | - | - | 0.00 (0.00–793.45) | 0/1/1 |
| Uranotaenia macfarlanei¹ | 0 (0.00–793.45) | 0/1/1         | - | - | 0.00 (0.00–793.45) | 0/1/1 |
| Total           | 5.35 (4.90–5.83) | 499/2848/102633 | 7.50 (6.82–8.22) | 439/1962/66666 | 1.73 (1.33–2.21) | 60/886/35967 |

¹Mosquito species captured at pig farms only.
²Mosquito species captured in wetlands only.
³The maximal likelihood estimation per 1000 mosquitoes and the 95% confidence interval (lower limit-upper limit).
⁴The number of JEV positive pools/the number of pools tested/the number of mosquitoes tested by RT-PCR.
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fuscocephala Theobald, were also reported as important JEV vectors [27–29], a relatively low number of these mosquitoes were captured, indicating that they play a minor role in the transmission of JEV in Taiwan.

The JEV infection rate of mosquitoes captured on the pig farms (7.50 per 1000) was significantly higher than the rate of those captured in the wetlands (1.73 per 1000) (p < 0.01), indicating that pigs played an important role in amplifying JEV. In addition, except for A. sinensis, the infection rates of all of mosquito species collected on the pig farms were higher than the rates of those in the wetlands (Table 2). In our study, JEV positive mosquitoes were captured in only one of the four wetlands, located in Beitou District, Taipei City. This wetland is near human habitats, where both water birds and pigs may serve as reservoirs or amplifying hosts for the JEV.

Interestingly, although 11 species of mosquitoes were RT-PCR positive for JEV, the virus was isolated solely from Cx. tritaeniorhynchus and Cx. annulus. Because both blood-fed and

Table 3. Summary of the maximum likelihood estimates (MLEs) of the mosquito Japanese encephalitis virus (JEV) infection rate by month, and the mosquito collection sites in the northern, central, southern, eastern, and all sites of Taiwan between 2005 and 2012.

| Month/Region | All sites | Northern | Central | Southern | Eastern |
|--------------|-----------|----------|---------|----------|---------|
| Mar          | 0 (0.00–65.51); 0/10/44 | ND² | ND | 0 (0.00–89.55); 0/5/27 | 0 (0.00–139.30); 0/5/17 |
| Apr          | 0 (0.00–1.05); 0/110/3586 | 0 (0.00–94.07); 0/4/24 | 0 (0.00–15.37); 0/12/207 | 0 (0.00–1.19); 0/80/3134 | 0 (0.00–14.67); 0/14/221 |
| May          | 4.98 (4.13–5.97); 113/696/24948 | 3.66 (2.43–5.31); 25/214/7227 | 4.45 (2.35–7.75); 11/77/2682 | 2.46 (1.62–3.60); 24/265/10220 | 14.66 (11.10–19.15); 53/140/4774 |
| Jun          | 6.72 (6.07–7.42); 370/1599/61889 | 7.09 (6.27–8.01); 249/1017/39692 | 7.80 (6.33–9.52); 92/337/13772 | 0.95 (0.39–1.98); 6/175/6382 | 12.08 (8.14–17.44); 23/70/2043 |
| Jul          | 1.46 (0.87–2.32); 16/385/11180 | 1.16 (0.43–2.57); 5/146/4422 | 2.47 (1.27–4.40); 10/137/4157 | 0 (0.00–1.69); 0/61/2186 | 2.35 (0.14–11.24); 1/41/415 |
| Aug          | 0 (0.00–3.71); 0/48/966 | 0 (0.00–19.15); 0/13/166 | 0 (0.00–12.93); 0/14/259 | 0 (0.00–6.22); 0/21/561 | ND |

Total 5.35 (4.90–5.83); 499/2848/102633 6.01 (5.34–6.74); 279/1394/51576 6.02 (4.99–7.22); 113/577/21077 1.37 (0.94–1.93); 30/607/22510 12.32 (9.82–15.32); 77/270/7470

¹The maximal likelihood estimation of the JEV infection rate per 1000 mosquitoes and the 95% confidence interval (lower limit-upper limit); and the number of JEV positive pools/the number of pools tested/the number of mosquitoes tested by RT-PCR.

²Not done.

Figure 2. Japanese encephalitis epidemic season in humans and MLE of the JEV infection rates in mosquitoes by month during 2005–2012.

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Table 4. Summary of the maximum likelihood estimates (MLEs) of the mosquito Japanese encephalitis virus (JEV) infection rate per year, and the mosquito collection sites (all sites, northern, central, southern, and eastern regions).

| Year/Region | All sites | Northern | Central | Southern | Eastern |
|-------------|-----------|----------|---------|----------|---------|
|             | IR (95%CI); No. positive pools/No. individuals¹ | No. GI/No. GIII² | IR (95%CI); No. positive pools/No. individuals | No. GI/No. GIII | IR (95%CI); No. positive pools/No. individuals | No. GI/No. GIII |
| 2005        | 12.12 (10.57–13.85); 206/742/20020 | 0/99 | 16.31 (13.70–19.31); 128/340/9810 | 0/39 | 6.06 (4.32–8.30); 36/218/6478 | 0/26 |
|             |           |         |         |         |         |         |
| 2006        | 5.35 (3.91–7.17); 42/336/659 | 0/42 | 10.32 (7.17–14.52); 31/92/3613 | 0/31 | 3.84 (1.97–6.68); 10/73/2785 | 0/10 |
|             |           |         |         |         |         |         |
| 2007        | 0.59 (0.11–1.93); 2/87/3410 | 0/2 | 5.04 (0.30–25.91); 1/7/202 | 0/1 | 0 (0.00–2.93); 0/31/1240 | 0/0 |
|             |           |         |         |         |         |         |
| 2008        | 11.04 (8.08–14.81); 42/159/454 | 2/40 | 19.45 (13.24–27.96); 28/64/1894 | 2/26 | 7.10 (2.93–14.86); 6/35/921 | 0/6 |
|             |           |         |         |         |         |         |
| 2009        | 2.05 (1.60–2.68); 65/801/33039 | 14/33 | 1.77 (1.27–2.40); 38/54/2326 | 6/15 | 3.56 (1.76–6.54); 9/61/2699 | 8/0 |
|             |           |         |         |         |         |         |
| 2010        | 3.27 (2.50–4.42); 56/482/18385 | 46/10 | 4.31 (2.60–6.78); 17/118/4355 | 17/0 | 7.13 (4.39–11.09); 18/75/2924 | 11/7 |
|             |           |         |         |         |         |         |
| 2011        | 6.01 (4.21–8.37); 63/146/6314 | 33/0 | 3.08 (1.72–5.15); 13/107/4522 | 13/0 | 15.87 (9.99–24.63); 20/39/1792 | 20/0 |
|             |           |         |         |         |         |         |
| 2012        | 7.54 (5.71–9.81); 53/195/8352 | 51/2 | 5.36 (3.49–7.94); 23/118/4854 | 21/2 | 7.35 (4.22–12.12); 14/45/2238 | 14/0 |
|             |           |         |         |         |         |         |
| Total       | 5.35 (4.90–5.83); 499/2848/102633 | 146/228 | 6.01 (5.34–6.74); 279/1394/51576 | 59/114 | 6.02 (4.99–7.12); 113/577/21077 | 53/49 |

¹The maximal likelihood estimation of the JEV infection rate per 1000 mosquitoes and the 95% confidence interval (lower limit-upper limit); and the number of JEV positive pools/the number of pools tested/the number of mosquitoes tested by RT-PCR.

²Numbers of Genotype I and genotype III JEV positive pools determined by RT-PCR and DNA sequencing.
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Figure 3. Proportions of genotype distributions of Japanese encephalitis virus strains and annual numbers of Japanese encephalitis cases in Taiwan between 2005 and 2012. The proportions of genotype distributions in northern (A), central (B), southern (C), eastern (D), all (E) of Taiwan, and the annual numbers of confirmed and death cases of individuals with Japanese encephalitis (F). NA = not available. doi:10.1371/journal.pntd.0003122.g003
| Species         | 2005     | 2006     | 2007     | 2005-2007 | 2008     | 2009     | 2010     | 2011     | 2012     |
|-----------------|----------|----------|----------|-----------|----------|----------|----------|----------|----------|
| Aedes aegypti   | 30.48 (2.28–138.68); 1/6/27 | 30.48 (2.28–138.68); 1/6/27 | 0.00 (0.00–97.86); 0/5/27 | 0 (0.00–499.14); 0/2/3 |
| Ae. albopictus  | 30.48 (2.28–138.68); 1/6/27 | 30.48 (2.28–138.68); 1/6/27 | 0.00 (0.00–97.86); 0/5/27 | 0 (0.00–499.14); 0/2/3 |
| Ae. vexans      | 31.04 (2.05–147.76); 1/5/30 | 30.34 (1.98–143.52); 1/6/31 | 14.92 (2.86–49.98); 2/16/141 | 0 (0.00–112.36); 0/3/19 |
| Ae. enguinensis | 0 (0.00–10.46); 0/10/283 | 0 (0.00–10.46); 0/10/283 | 0 (0.00–10.46); 0/10/283 | 0 (0.00–10.46); 0/10/283 |
| Ae. vexans      | 0 (0.00–793.45); 0/1/1 | 0 (0.00–793.45); 0/1/1 | 111.48 (27.79–316.17); 2/5/23 | 0 (0.00–165.40); 0/5/28 |
| Anopheles ludsowae | 0 (0.00–793.45); 0/1/1 | 0 (0.00–793.45); 0/1/1 | 14.86 (1.08–13.47); 0/2/64 | 0 (0.00–20.65); 0/5/12 |
| Coquilletidae    | 0 (0.00–35.21); 0/1/44 | 0 (0.00–35.21); 0/1/44 | 10.68 (0.70–49.65); 1/14/85 | 0 (0.00–20.65); 0/5/12 |
| Culex annulatus | 21.49 (9.04–48.19); 5/50/127 | 22.91 (1.49–112.23); 1/5/41 | 111.48 (27.79–316.17); 2/5/15 | 0 (0.00–165.40); 0/5/14 |
| Cx. bitaeniorhynchus | 0 (0.00–20.11); 0/5/12 | 0 (0.00–20.11); 0/5/12 | 14.86 (1.08–13.47); 1/2/64 | 0 (0.00–29.48); 0/7/9 |
| Cx. brevipalpis | 0 (0.00–793.45); 0/1/1 | 0 (0.00–793.45); 0/1/1 | 14.86 (1.08–13.47); 1/2/64 | 0 (0.00–29.48); 0/7/9 |
| Cx. fuscanus     | 0 (0.00–657.62); 0/2/2 | 0 (0.00–657.62); 0/2/2 | 0 (0.00–20.65); 0/5/12 | 0 (0.00–29.48); 0/7/9 |
| Cx. fuscocephala | 13.01 (3.96–47.66); 2/5/110 | 13.01 (3.96–47.66); 2/5/110 | 0 (0.00–20.65); 0/5/12 | 0 (0.00–20.65); 0/5/12 |
| Cx. mimeticus   | 0 (0.00–793.45); 0/1/1 | 0 (0.00–793.45); 0/1/1 | 0 (0.00–16.35); 0/12/195 | 0 (0.00–16.35); 0/12/195 |
| Cx. murelli     | 0 (0.00–793.45); 0/1/1 | 0 (0.00–793.45); 0/1/1 | 0 (0.00–16.35); 0/12/195 | 0 (0.00–16.35); 0/12/195 |
| Cx. nigropunctatus | 0 (0.00–793.45); 0/1/1 | 0 (0.00–793.45); 0/1/1 | 0 (0.00–16.35); 0/12/195 | 0 (0.00–16.35); 0/12/195 |
| Cx. quinquefasciatus | 4.58 (0.84–15.05); 2/24/446 | 4.10 (0.75–13.47); 2/29/496 | 0 (0.00–16.35); 0/12/195 | 0 (0.00–16.35); 0/12/195 |
| Cx. sitiens     | 0 (0.00–0.00); 0/37/745 | 0 (0.00–0.00); 0/37/745 | 0 (0.00–16.35); 0/12/195 | 0 (0.00–16.35); 0/12/195 |

Table 5. Summary of the mosquito species, the maximum likelihood estimates (MLEs) of the mosquito Japanese encephalitis virus (JEV) infection rates by year.
This study demonstrated the intense JEV transmission activity in Taiwan and highlights the importance of JE vaccination to control this epidemic. Continuous monitoring of the JEV strain variations and their gene sequence evolution can provide valuable information for the assessment of the vaccine's efficacy.
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Author Contributions

Conceived and designed the experiments: CLS HJT PYS. Performed the experiments: CLS CFY LCL CL KHT YYC LYC SFC. Analyzed the data: CLS CFY HJT KHT PYS. Contributed reagents/materials/analysis tools: PYS. Wrote the paper: CLS HJT KHT PYS.

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