Materials and Methods

1. Mosquito collection in waterfowl protected areas

The Wu-Wei Gang waterfowl protected areas in Yilan, northern Taiwan were visited to collect mosquitoes on May and September to study the transmission of avian malaria parasites and flavivirus through migrating birds. All mosquitoes were carried back to laboratory by dry ice to identify the mosquito species and sex by following Lien's key (Lien 2004). After species identification, *Culex tritaeniorhynchus* Giles were stored at a -80°C freezer in laboratory of Taiwan CDC for further detection of flavivirus, including Japanese Encephalitis virus. The maximal number of mosquitoes tested per pool was 50. Other mosquitoes were brought back to NIID laboratory for further detection on avian malaria parasites.

2. Virus detection of flavivirus and JE virus in mosquitoes

The detailed procedure was described in papers published by Huang et al. (2010) and Yang et al. (2010). Briefly, Mosquito pools were homogenized by tissue lyser and clarified by centrifugation. Viral RNAs (70μL) were extracted from 140μL of mosquito suspension, using the QIAamp viral RNA mini kit (Cat. No.52906, Qiagen, Hilden, Germany) according to the manufacturer's instructions. A one-step SYBR Green I-based Real-Time Reverse Transcription-PCR Assay was used to test for Japanese Encephalitis virus and Flavivirus infection .RT-PCR amplification was performed in the LightCycler 480 II PCR system (Roche, Switzerland). Mosquito pools were assayed in a 50μL reaction mixture containing 10μL of sample RNA and viral specific primers by using QuantiTect SYBR Green RT-PCR kit (Cat. No.204243, Qiagen, Hilden Germany). Qiagen). Two sets of primers including Flavivirus-specific primers (FL-F1: 5'-GCCATA TGG TAC ATG TGG CTG GGA GC-3'; FL-R3: 5'-GTK ATT CTT GTGTCC CAW CCG GCT GTG TCA TC-3; FL-R4: GTG ATG CGR GTG TCCCAG CCR GCK GTG TCA TC-3') and JE virus-specific primers (10F : 5'-CTG GGA ATG GGC AAT CGT G-3', 325R:5'-TGT CAA TGC TTC CCT TCC C-3') were used for real-time RT-PCR. The former (FL-F1, FL-R3 and FL-R4) targeted a consensus region of the nonstructural protein 5 (nsp5) genes to detect all Flaviviruses and the products were expected to be 212 bp. The latter (10F and 325R) targeted a consensus region of the nonstructural protein 1 (nsp1) genes to detect all JE virus, and the products were expected to be 334 bp. The thermal program consisted of a 30-min RT step at 50°C and 15 min of Taq polymerase activation at 95°C, followed by 45 cycles of PCR. Following amplification, a melting curve analysis was performed to verify the correct product by its specific melting temperature.

3. Information sharing on dengue, JE, and mosquito collections

Information sharing on epidemiology of dengue and JE are included in this project through CDC web site (http://www.cdc.gov.tw). Dengue and JE are classified as notifiable diseases in Taiwan. They require physicians to report the infection within 24 hours and 7 days of clinical diagnosis, respectively. Additionally, fever screening at airports in Taiwan was launched as part of active surveillance for a panel of notifiable infectious diseases, including dengue (Shu et al., 2005). The arriving passengers are screened for fever by infrared thermal scanners. Later, the airport clinicians evaluate the passengers with fever for further diagnostic testing decision.

(1) Clinical samples and laboratory diagnosis

Human serum samples from clinically suspected DENV and other arbovirus infections were submitted to Research and Diagnostic Center, Centers for Disease Control, Taiwan (Taiwan CDC), Department of Health, for laboratory diagnosis. A confirmed dengue case was defined as febrile illness associated with a positive real-time reverse transcription (RT)-PCR test, isolation of DENV, or a ≥4-fold increase in DENV-specific IgM and IgG antibodies. A multiplex one-step real-time RT-PCR was developed to simultaneously detect and differentiate various flaviviruses and alphaviruses in the acute-phase serum samples using group-specific and virus-specific primers. In addition, a flavivirus/alphavirus-specific capture ELISA was developed to detect and differentiate various flavivirus/alphavirus infections (Chen et al. 2010, Shu et al. 2003 a, b).

(2) Virus isolation and identification

DENVs were isolated from the acute phase serum samples of confirmed cases. The virus isolation was performed using mosquito cell line (clone C6/36 of Aedes albopictus cells). For each acute phase serum, 4μL of serum sample was diluted in 200μL cultured medium （RPMI , Gibco/BRL, Life Technologies, containing 1% FCS） and added to a 96-well microtiter plate, 50μL/well in quadruplicate. Then, 105 cells/100μL well of C6/36 cell line were added into the microtiter plate and incubated at 30°C for 2-7 days. Cells were harvested and virus isolates were identified by the indirect fluorescent antibody test with virus group-specific and serotype-specific monoclonal antibodies.

(3) Primers used for RT-PCR and nucleotide sequencing of DENV

The diagnostic tests for flavivirus infection from fevered patients on the basis of the results of one-step SYBR Green I-based real-time RT-PCR and envelope/membrane-specific capture IgM and IgG ELISA had been described previously (Chen et al. 2010, Shu et al. 2003 a, b). To screen viremic fever patients with alphavirus infection, a multiplex one-step SYBR Green I-based real-time RT-PCR was developed. A cocktail consisted with three sets of primers were mixed and used for RT-PCR screening. The alphavirus-specific primer set (AL-2: 5'-AAG CTY CGC GTC CTT TAC CAA AG-3'and AL-3: 5'-GTG GTG TCA AAC CCT ATC CA-3') targeted a consensus region of the nonstructural protein 1 (nsp1) genes to detect all alphaviruses (Shu et al. 2008). Positive results were then confirmed by gene sequence analysis, virus isolation, and serological test.

(4) Preparation of viral RNA, RT-PCR amplification and nucleotide sequencing

Viral RNAs were extracted from either acute phase serum samples or culture supernatant of C6/36 cell line infected with each of the isolated DENV or CHIKV strains using the QIAamp viral RNA mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Nucleotide sequences of partial NS5 gene fragment of DENV were determined directly from acute phase serum samples using RT-PCR product of one-step SYBR Green I quantitative RT-PCR . Partial NS5 gene sequencing was routinely performed to detect and differentiate serotype and genotype of the newly identified DENVs. For full-length structure gene sequencing, extracted viral RNA from culture supernatant of C6/36 cell line infected with each of the isolated DENV strains was used as a template for cDNA synthesis, which subsequently was used for PCR amplification. Two overlapping PCR products spanning the full-length structure gene were purified from agarose gels and directly sequenced in both directions using ABI Prism automated DNA sequencing kit and ABI Prism 3700 DNA sequencer (Applied Biosystems) according to the manufacturer's protocol. Overlapping nucleotide sequences were combined for analysis and edited with the Laser software package (DNASTAR Inc, Madison, WI) (Huang et al. 2005, Shu et al. 2009).

(5)Phylogenetic analysis

Phylogenetic analyses were conducted using PHYLIP (version 3.6) or MEGA version 5. Genetic distances were calculated by using Kimura 2-parameter distance algorithm with 1,000 bootstrap replicates. Neighbor-Joining method was used to generate the phylogenetic trees.

Results

Virus detection in mosquitoes

A total of 497 C*x. tritaeniorhynchus* females adults were grouped by date into 11 pools (Table 1). Among these pools, 6 contained 50 mosquitoes, 1 contained and 1 contained. This 11 pools were analyzed for both flavivirus and JE virus infections by the fluorogenic real-time PCR and all were negative (Table 1, Figure 1 and 2). The fluorescence intensity of positive control (single female *Cx. tritaeniorhynchus* plus JE Virus 5μL) started to increase at cycle 27-28 and cycle 22-23, respectively.

Information sharing

The numbers of dengue cases and JE cases by weeks, months, year, and location from 1998 to 2010 in excel format can be download from Taiwan CDC' web site (http://nidss.cdc.gov.tw/SingleDisease.aspx?Pt=s&Dc=1&Dt=2&disease=061) to provide NIID for further global warming study. Additionally, epidemiological trend (Figure 3 and 4) and geological distributions of cases (Figure 5 and 6) were also available. The data on 2011 is presented as followings:

1. Imported dengue cases in Taiwan, 2011

A total of 157 laboratory confirmed imported dengue cases were identified in Taiwan, 2011. Table 2 showed the summary of countries of origin and DENV serotypes of imported cases. The imported cases were arriving from 10 countries. Most of these imported cases were infected in Vietnam, the Philippines, Indonesia, and Thailand. DENV-1 and DENV-2 contained the predominant serotypes of DENV strains. Among them, 31 DENV-1, 26 DENV-2, 9 DENV-3, and 11 DENV-4 strains were isolated. The nucleotide sequences of complete E gene sequences of DENV strains were determined and deposited in Taiwan Pathogenic Microorganism Genome Database for molecular epidemiological analysis.

2. Multiple dengue epidemics in Taiwan, 2011

For local dengue outbreaks in Taiwan, a total of 1545 dengue patients were laboratory confirmed with 22 cases of DHF in 2011. The complete E gene sequences of DENV strains isolated from representative indigenous dengue cases were determined. Representative indigenous cases were selected based on the information of epidemiological investigation and preliminary results of partial NS5 gene sequences showing different infection time, infection place, and DENV serotypes and genotypes of these patients. Sequence analyses of DENV isolates obtained from acute phase serum samples of indigenous cases showed that 9 different DENV strains (2 DENV-1, 4 DENV-2, and 3 DENV-3) were circulated in Kaohsiung City (1168 cases), Pingtung County (149 cases), Penghu County (98 cases), Tainan City (95 cases), and Taipei and New Taipei Cities (28 cases). Notably, no dengue outbreaks have been

recorded in Penghu County since 2002, however, a DENV-2 outbreak occurred in this County in 2011 with at least 98 cases.

3. Nucleotide sequencing and phylogenetic analysis

Phylogenetic analyses of E gene sequences of DENVs isolated from indigenous and imported dengue cases showed that epidemic DENVs circulating in Taiwan in 2011 might be introduced from Vietnam, Indonesia, Singapore, Malaysia, Myanmar, the Philippines, and Central Americas. Figure 7 showed the phylogenetic tree derived from complete E gene sequences of DENV-2. Phylogenetic analysis showed that DENV-2 strains isolated from imported cases from Vietnam and Thailand in 2011 belonged to Asian genotype 1. DENV-2 strains isolated from imported cases from Singapore and the Philippines belonged to Cosmopolitan genotype. A DENV-2 strain, D2/Taiwan/802KH1108c/2011, which belonged to Asian genotype 1, is the major epidemic strain circulating in Kaohsiung City and Penghu County in 2011. This strain is closely related to virus strains from Vietnam. Figure 8 shows the phylogenetic tree of complete E gene sequences of DENV-1 strains. Phylogenetic analysis showed that genotype I of DENV-1 contains virus strains isolated from imported cases from Indonesia, Malaysia, Myanmar, Cambodia and Vietnam. Genotype II of DENV-1 contains virus strains from the Philippines. Genotype III of DENV-1 contains virus strains from India and Bangladesh. A DENV-1 strain, D1/Taiwan/700TN1109a/2011, is the major epidemic strain circulating in Tainan City in 2011. Notably, this strain belonged to genotype III and is closely related to virus strains from Haidi in Central America.

In mosquito collections, two mosquito collection techniques were learned from Dr. Yoshio Tsuda. One is the portable light traps designed by Dr. Yoshio Tsuda for travel convenience purpose (Figure 7). The other is using paper cups and stocking socks to collect mosquitoes and to keep the mosquitoes alive by folding socks with sugar solution cotton pads inside.

Discussion

The frequency of dengue outbreak occurred has increased for the past 5 years and the high number of JE vectors and malaria vectors were found in Taiwan. Additionally, new introductions of chikungunya virus cases occurred through travelers from endemic countries to this island. Therefore, the threat of these mosquito-borne diseases is increasing. The control strategy for each disease varies. For example, introductions of dengue virus cases occurred so frequently each year that lower vector densities should be prioritized. The pathogen introductions for malaria are less frequent and their vectors have limited distributions and unstable populations; therefore, this disease should focus on patient diagnosis and management.

The increase in international trade and tourism facilitated the spread of disease vectors and pathogens. Our results showed the constant importation of various DENV strains into currently non-endemic areas, including Japan and Taiwan. Molecular epidemiologic studies analyzing DENV strains isolated from imported and indigenous cases showed that different serotypes, genotypes, and/or strains were responsible for outbreaks that occurred each year, and the epidemic strains disappeared with the ending of each local outbreak in Taiwan. Notably, our study showed that epidemic strains circulating in Taiwan were all introduced from Western Pacific and Southeast Asia, except an epidemic strain that circulating in Tainan City in 2011 belonged to genotype III of DENV-1, which is closely related to virus from Central America. The results demonstrated that local mosquitoes in Taiwan are competent vectors for transmission of a variety of DENV strains circulating in Southeast Asian, Western Pacific as well as Central America. More effective and efficient control program of dengue, including various surveillance systems, network of rapid diagnostic laboratories, and rapid response carried out by central and local health departments would be needed to control dengue and maintain Taiwan as a non-endemic country.

In Taiwan, *Culex tritaeniorhynchus* was the dominant species among JE vectors in Taiwan, accounted for 64.1% to 100.0% of total number of JE vectors. Its population reached the peak during June-September. JE virus became active among field mosquito population during May-June, which occurred earlier (in May) in southern Taiwan and later (in June) in Taipei. JE virus activity was coincidence with the minor peak of JE vector density, not the major peak (Lin et al. 2010). The scenario regarding JE virus activities indeed corresponds to the occurrence of JE human cases (generally started at May, peak in June or July). Previous study indicated that the duration of viremia of JE virus in pigs or birds was 2-5 days. The infected JE vectors become infectious 5-1days after blood-feeding of virus and maintain the transmissibility during their lifetime. The average survival time for female *Cx.*

tritaeniorhynchus was 19.2 days (Reisen et al. 1979). During April-June, the JE mosquito density displayed in an increasing trend and the JE virus largely amplified inside pigs in Taiwan. Subsequently, pigs develop antibody to JE virus and, thus, mosquitoes will no longer contract infection from these pigs. Therefore, it was not surprising that mosquitoes collected were all negative in virus infection. This analysis included one year data only.

Acknowledgements

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Publication list for this work

NIL

Table 1. Virus infection in field-caught *Culex tritaeniorhynchs* adults collected in Wu-Wei Gang waterfowl protected areas in Yilan on May and September, 2011

Virus detected	Total pools tested	Positive pools	No. of *Culex tritaeniorhynchus* tested	
			May	September
JE virus	11	0	39	458
Flavivirus	11	0	39	458

Table 2. Countries of origin and DENV serotypes of imported cases in 2011, Taiwan

Country of origin	Total cases	DENV Serotype				
		DENV-1	DENV-2	DENV-3	DENV-4	unknown
Vietnam	34	10	7	1	1	15
Philippines	34	8	9	4	5	8
Indonesia	25	3	6	5	3	8
Thailand	21	1	9		1	10
Malaysia	13	1	2	2	2	6
India	8	2	1	3		2
Cambodia	8	5				3
Bangladesh	6	1		1		4
Singapore	4	1	3			
Myanmar	4	1			1	2
Total	157	33	37	16	13	58

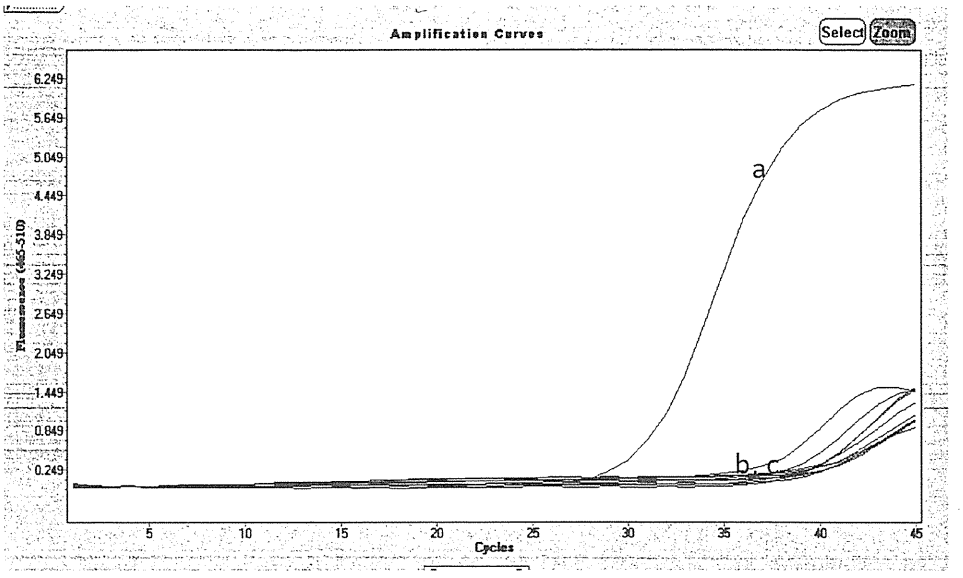

Figure 1. Results from fluorogenic real-time PCR amplifications of flavivirus in *Culex tritaeniorhynchus* specimens collected in Yilan Township, Yilan Countyin May and September 2010. a: positive control in entire process (single female *Cx. tritaeniorhynchus* , JEV 5μL), b: negative control in entire process (single female *Cx. tritaeniorhynchus*), c: no-template negative control in PCR assays, other background curves: negative results of 11 pools of *Cx. tritaeniorhynchus* specimens.

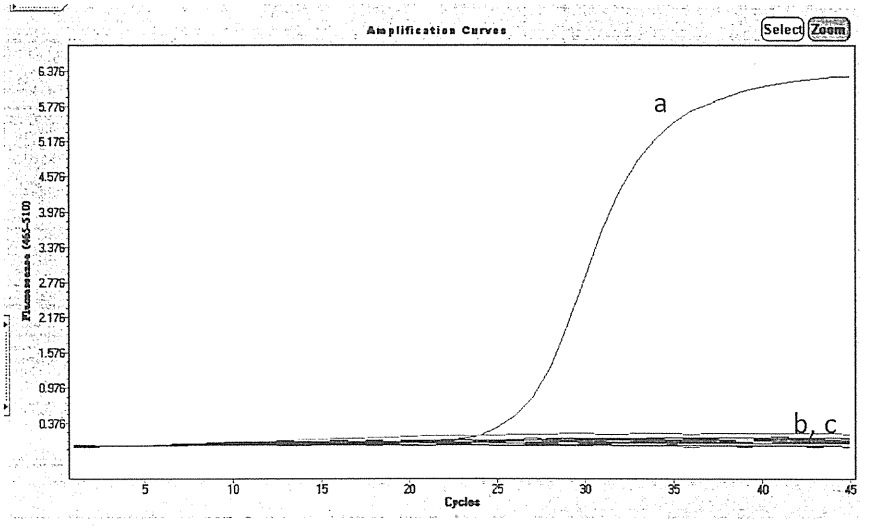

Figure 2. Results from fluorogenic real-time PCR amplications of Japanese encephalitis virus in *Culex tritaeniorhynchus* specimens collected in Yilan Township, Yilan Countyin May and September 2010. a: positive control in entire process (single female *Cx. tritaeniorhynchus* , JEV 5μL),b: negative control in entire process (single female *Cx. tritaeniorhynchus*), c: no-template negative control in PCR assays nd curves: negative results of 11 pools of *Cx. tritaeniorhynchus* specimens.

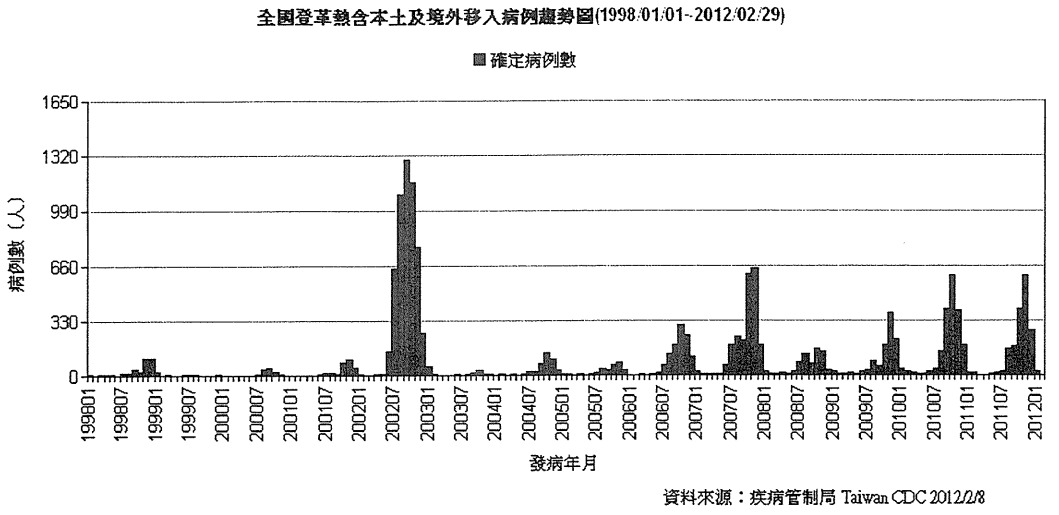

Figure 3. The number of Dengue cases per month from 1998 to feb. 2012 in Taiwan.

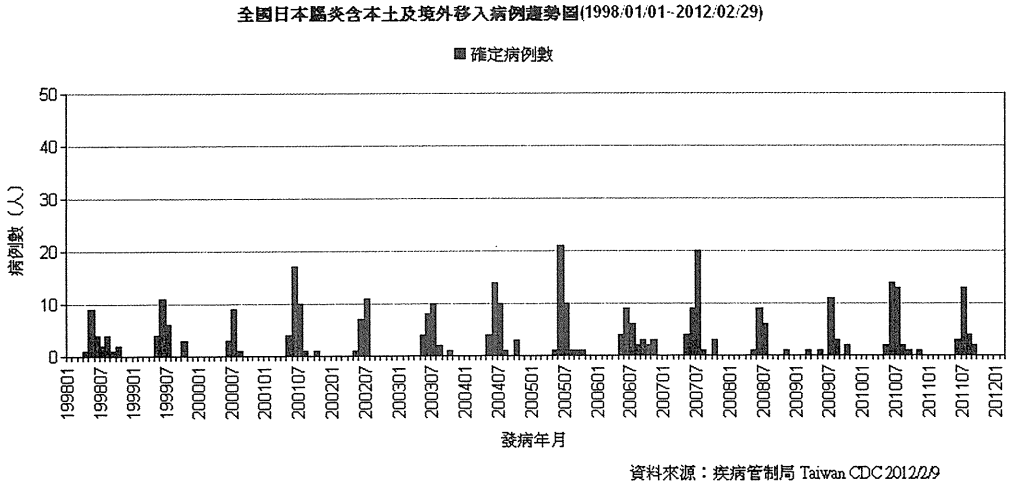

Figure 4. The number of Japanese Encephalitis cases per month from 1998 to feb. 2012 in Taiwan.

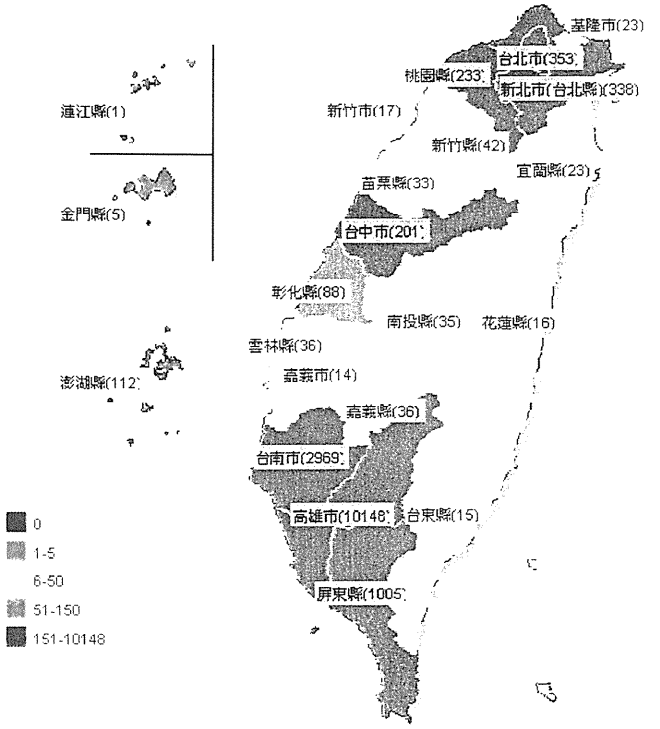

Figure 5. The dengue distribution map by counties/cities from 1998 to feb. 2012 in Taiwan.

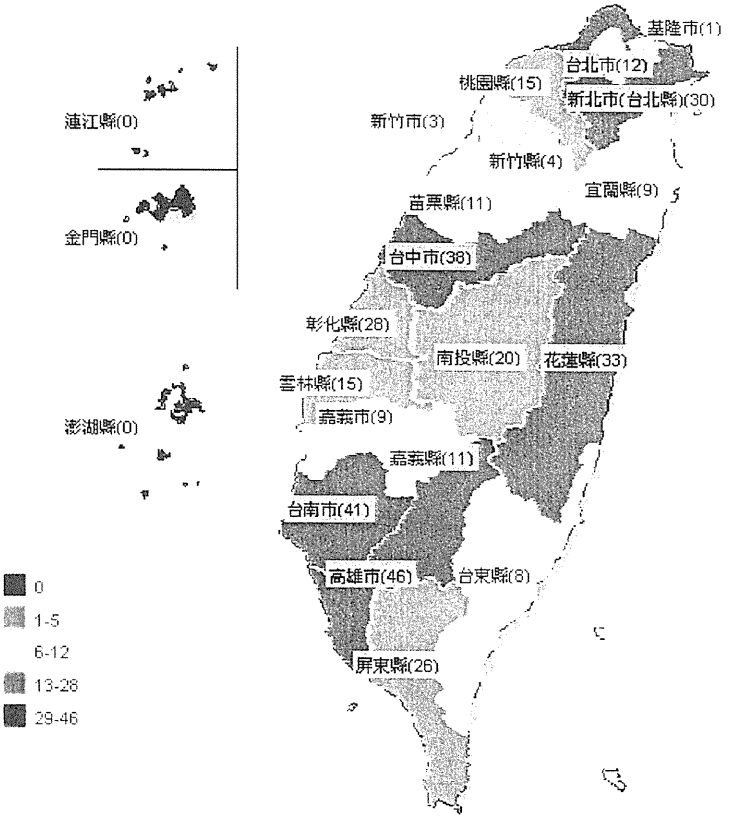

Figure 6. The JE distribution map by counties/cities from 1998 to feb. 2012 in Taiwan.

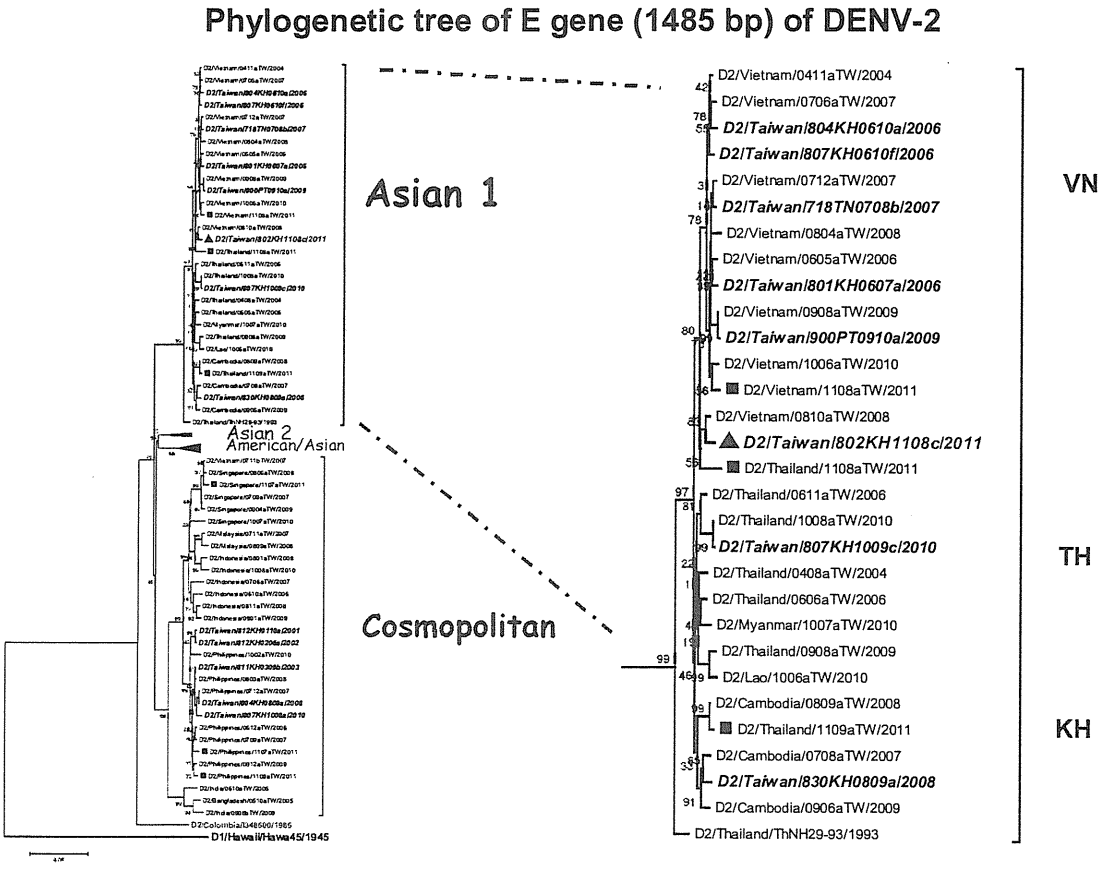

Figure 7. Phylogenetic trees derived from full-length E gene sequences of DENV-2. DENV-2 strains isolated from imported and indigenous cases in Taiwan, 2011, are designated in full squares and full triangles, respectively. The trees were constructed by the neighbor-joining method. Viruses were identified using the nomenclature of serotype/country/strain/year of isolation.

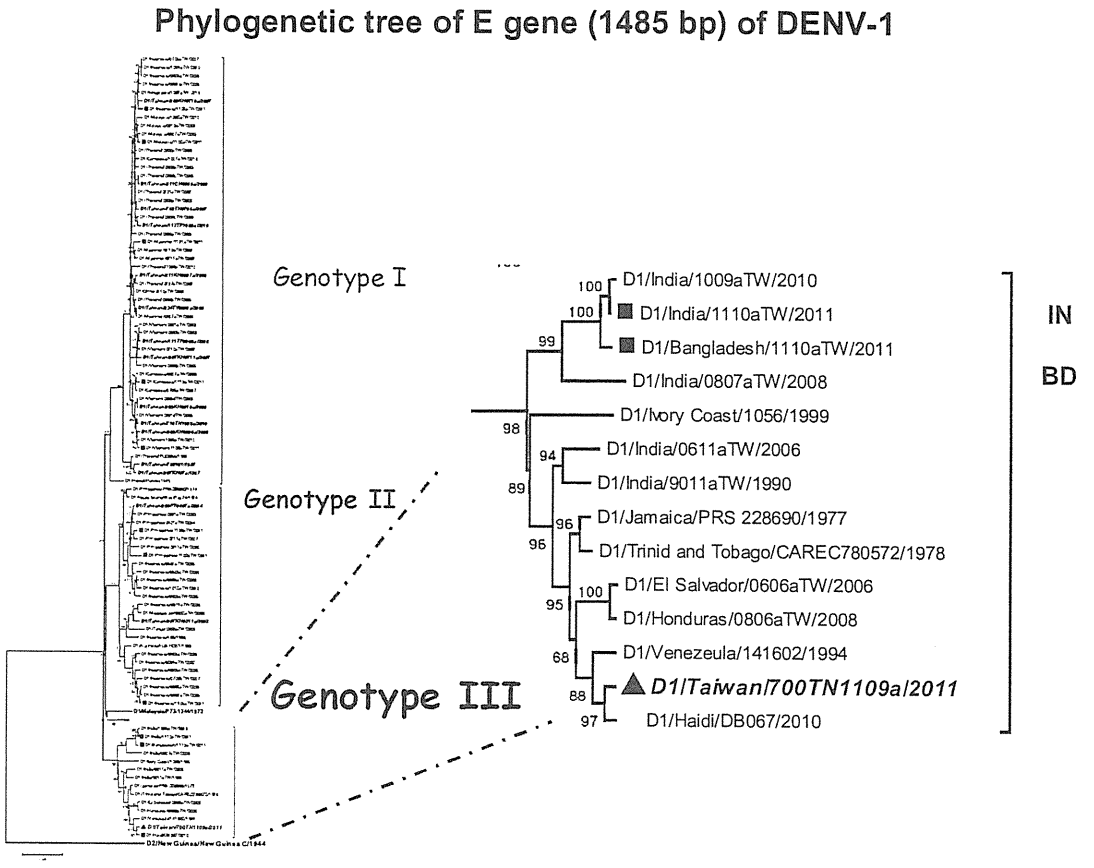

Figure 8. Phylogenetic trees derived from full-length E gene sequences of DENV-1. DENV-1 strains isolated from imported and indigenous cases in Taiwan, 2011, are designated in full squares and full triangles, respectively. The trees were constructed by the neighbor-joining method. Viruses were identified using the nomenclature of serotype/country/strain/year of isolation.

Figure 9. A portable light trap designed by Dr. Yoshio Tsuda used in mosquito collection, Ilan, Taiwan.

Genetic analysis of highly virulent strains of *Entamoeba histolytica* in the high risk groups between Taiwan and Japan

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Keywords: *Entamoeba histolytica*; molecular typing; tRNA-linked short tandem repeats (STRs); Men Who Have Sex with Men (MSM); phylogenetic analysis; cluster; Unweighted Pair Group Method with Arithmetic Mean (UPGMA); AIG1 protein gene

Abstract

Amoebiasis is still an important parasitic disease resulting in severe and invasive disease all over the world and causing about 100 thousands death each year. In Taiwan, there are four risk groups: foreign workers, HIV/Men Who Have Sex with Men (MSM), institutionalized patients and aboriginals. The cases of amoebiasis are slowly increasing in foreign worker and HIV/MSM groups, but sporadically outbreak in institutionalized patient group in recent years. However, not all of *Entamoeba histolytica* strains could cause disease. Study of the strain variations could reveal their genetic connection and transmission pattern among humans. The objective of this study is to develop a phylogenetic assay of *E. histolytica* strains among different geographic areas. In 2009, a total of 93 PCR confirmed cases were grouped into Taiwanese patients and foreign workers. Six tRNA-linked short tandem repeats (STRs) loci were PCR amplified and sequenced and then used for the phylogenetic analysis by UPGMA method and minimum spanning method. Compared to the sequences of *E. histolytica* strains from Indonesians, Filipinos, Vietnamese, and Japaneses, the Taiwanese strains were closer to Japanese, some even genotype identical. Several strains of mental patients from Japan were clustered together with Taiwanese strains of mental patients. Furthermore, the strain clusters might also relate to clinical symptoms and risk groups. Two different genotypes were found in two phylogentically unrelated HIV/MSM groups. Stains from same institution were usually clustered together. This study may provide a new understanding in the amebic genotypes, geographic origins and clinical symptoms among high risk groups, which may be used in further disease treatment and control.

Introduction

The severity of amoebiasis is only less than malaria in parasite diseases. *Entamoeba histolytica* is the etiologic agent of amebiasis with approximately 50 million cases and 100,000 deaths each year (1, 2). *E. histolytica* infection of the colon results in dysentery, ulcers, and colitis. Trophozoites can invade the colon epithelium and disseminate to the liver or other soft organs to form abscesses. The disease is spread primarily by cysts in contaminated food or water, but may also be transmitted from person to person. Recent reports describe high prevalence of invasive amebasis in men who have sex with men (MSM) in developed countries such as Australia, Japan, Korea, and Taiwan (3, 4, 5, 6). In Taiwan, *E. histolytica* infections in patients of psychiatric rehabilitation institutes, travelers returning from epidemic areas, alien workers, and foreign brides have been reported (7, 8, 9).

Microscopic examination of stool smears is used routinely in hospitals to distinguish *E. histolytica* from nonpathogenic species such as *E. coli*, *E. polecki*, *E. hartmani*, *Iodamoeba buetschlii*, and *Endolimax nana*. However, *E. histolytica*, *E. dispar*, and *E. moshkovskii* are morphologically identical and cannot be differentiated by microscopy (10). Consequently, alternative diagnostic approaches such as ELISA or PCR or multiplex real-time PCR (11, 12, 13) were developed for their differential diagnosis. Nevertheless, not all *E. histolytica* infections can cause disease in the human host. Only about 1 in 10 *E. histolytica* infections may progress to the development of clinical symptoms (14). What determines the outcome of an *E. histolytica* infection is still a mystery, but one possibility is that it is linked to the genotype of the parasite. To investigate this relationship, a simple, sensitive, and reliable method for strain identification is required (15).

Since the genome sequence of *E. histolytica* HM-1:IMSS strain was completed