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Presence of Japanese Encephalitis Virus Vectors in Can Tho City

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Förekomst av vektorer för Japanskt encefalitvirus i Can Tho city

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ABSTRACT

Japanese encephalitis virus (JEV) is a significant vector-borne zoonotic pathogen, causing devastating encephalitis in humans. Its geographical range includes a majority of Asian countries and has also been recognized in some western Pacific areas. The main vectors of JEV are mosquitoes belonging to the genus *Culex*. Birds and pigs function as hosts and virus amplifiers, whereas humans are accidental hosts. Japanese encephalitis is commonly regarded as a rural disease. In Vietnam, increased urbanization in combination with intensification of agricultural practices and urban animal keeping may cause JEV to become an increasing concern in urban areas. This study investigated stagnant waters as possible urban breeding sites for JEV vectors in Can Tho city, Vietnam. Mosquitoes were collected by hand net at dusk and larvae were collected from the waters. Specimens were subsequently identified and analysed for presence of JEV using a nested RT- PCR. A total number of 4,110 mosquitoes and 368 larvae were collected, the vast majority belonging to the *Culex* genus. The initial RT-PCR results showed 13 positive (of 130 tested) pools of mosquitoes, no positive larvae were found. The results indicate that different types of stagnant waters may serve as breeding grounds for JEV vectors in cities and are conceivably important in the urban epidemiology of JEV. In addition a small survey was carried out focusing on people's awareness of Japanese encephalitis, vaccination status and mosquito prophylaxis. Both native Vietnamese and tourist/foreigners were asked to participate.

SAMMANFATTNING

Japanskt encefalitvirus (JEV) är en viktig vektorburen zoonotisk patogen som orsakar allvarlig encefalit hos människor. Viruset är spritt i stora delar av Asien samt vissa områden i västra Stilla havet. Myggor tillhörande genuset *Culex* är de huvudsakliga vektorerna. Fåglar och grisar är värddjur för JEV och uppförökar viruset, medan människor är tangentiella värddjur. Japansk encefalit anses i huvudsak vara en landsbygdssjukdom. I Vietnam sker en ökad urbanisering och en intensifiering av jordbruket, vilket kan leda till att JEV blir ett ökande problem även i städer. Denna studie undersökte stillastående vatten som möjliga föryngringsplatser för JEV-vektorer i Can Tho city i Vietnam. Myggor fångades med håv vid skymning och mygglarver samlades från vattenansamlingarna. De insamlade exemplaren identifierades och analyserades för innehåll av JEV med en nestad RT-PCR. Totalt fångades 4,110 myggor och 368 larver, majoriteten tillhörande genuset *Culex*. RT-PCR resultat visade på 13 positiva (av 130 testade) myggpooler men inga positiva mygglarvspooler. Resultaten indikerar att olika typer av stillastående vatten utgör fortplantningsplatser för JEV-vektorer i städer och kan således vara en viktig del i virusets urbana epidemiologi. Därutöver genomfördes en mindre enkätundersökning som fokuserade på människors medvetenhet om Japansk encefalit, vaccinationsstatus och användande av myggprofylax. Både vietnameser och turister/utlänningar tillfrågades.

TABLE OF CONTENTS

INTRODUCTION

Japanese encephalitis virus (JEV) is a vector-borne zoonotic flavivirus, that can cause serious and, at times, fatal encephalitis in humans (Solomon et al. 2000). It is the most common mosquito-transmitted pathogen causing encephalitis worldwide (Weaver and Reisen 2010).

JEV is widespread in a majority of Asian countries as well as and in some western Pacific areas (Hills 2011), encompassing almost half of the world's population (Erlanger 2009). JEV was recently estimated to cause over 65,000 cases of encephalitis each year (Campbell et al. 2011). Due to lack of data and underreporting, estimates are rough and some earlier estimates have been as high as 125,000 cases annually (Tsai 2000). About 75% of all cases are children in the age group 0-14 years (Campbell et al. 2011).

A stable level or even decline in Japanese encephalitis (JE) incidence has been seen in countries where surveillance and vaccination programs have been successfully implemented, such as Japan, Thailand and China. In contrast an increasing number of cases are seen in other countries such as Cambodia, India and Laos, where such measurements have not been taken (Erlanger 2009, Tseng et al. 2003).

Mosquitoes from the genus *Culex* and in particular *Culex tritaeniorhynchus* are the most important vectors of JEV (Buescher et al. 1959b, Rosen 1986). Birds and pigs serve as reservoirs and amplifiers of the virus whereas humans are understood to be accidental dead-end hosts (Solomon et al. 2000, Erlanger 2009). JEV can cause reproductive disturbances in pigs and encephalitis in horses (SJV 2008).

The spread of JEV to new areas may be attributed to both the growth of the human population and intensification and structural changes in agricultural practices. Pig farms provide virus reservoirs and amplifiers, while rice paddies and irrigation systems make up breeding grounds for vectors (Erlanger 2009). The effects that climate changes may have on disease transmission are difficult to predict, but may influence both vector and host factors (Githeko et al. 2000).

In Vietnam, as in large parts of Asia, the agricultural sector is rapidly growing. Vietnams production of pork is continuously escalating, but it is still to a large extent relying on small household farms (Tisdell 2008). Lindahl (2012) suggests that JE may become an increasing concern in cities, as increased urbanisation combined with a need to keep livestock (in particular pigs) creates new conditions for urban disease transmission.

This study evaluates the prevalence and proportion of JEV vectors in Can Tho city, in the Mekong delta region of Vietnam. Presence of JEV transmission in Can Tho city has previously been demonstrated (Lindahl 2012). The purpose of this study was to further elucidate the epidemiology of JEV in an urban setting by examining stagnant pools of waters as possible urban breeding grounds for JEV vectors. This was achieved by collecting larvae and mosquitoes at 20 different sites in the most urban districts of Can Tho city. The collected specimens were identified and subsequently analysed for JEV using a nested reverse transcriptase polymerase chain reaction (RT-PCR).

In addition, a small survey was carried out to gain information on awareness of JE, vaccination status and precautions taken to avoid mosquito bites, targeting both native Vietnamese people and tourists visiting Vietnam.

LITTERATURE OVERVIEW

Japanese encephalitis virus

JEV belongs to the family *flaviviridae,* which in turn is divided into three genera; hepaciviruses, pestiviruses and flaviviruses. JEV is grouped into the later, together with other notable flaviviruses such as Yellow fever virus, Tick-Borne encephalitis virus, Dengue virus and West Nile virus. Most flaviviruses are so called arboviruses (i.e. they are transmitted via arthropod vectors to vertebrates) (Unni et al. 2011). Of the know flaviviruses about half are transmitted by mosquitoes, the rest are either tick-borne or without known vectors (King et al. 2011). JEV is further divided into 5 genotypes (Uchil and Satchidanandam 2001).

Flaviviruses are small, approximately 50nm in diameter, enveloped positive-sensed single-stranded RNA viruses with a spherical shape. Mature virions have three structural proteins; C (capsid) and the membrane-associated M (membrane) and E (envelope). Immature virions instead have prM (precursor membrane) which is later cleaved into M. The envelope protein mediates receptor binding (necessary for endocytosis) and fusion activity. In addition, there are seven non-structural proteins produced by infected cells (King et al. 2011). The length of the RNA genome is approximately 11kb (Unni et al. 2011).

Epidemiology

JEV is a zoonotic agent that is spread between vertebrate hosts via mosquitoes. Wading birds (e.g. herons and egrets) serve as reservoirs and pigs as amplifiers of the virus. In general they show no clinical signs but develop a high viremia, which allows the virus to infect mosquitoes during feeding (Buescher et al. 1959a). Humans and other vertebrates appear to be dead-end hosts, not producing a viremia substantial enough to pass the infection on (Solomon et al. 2000, Mackenzie et al. 2004).

JEV is found throughout southern and eastern Asia (van den Hurk et al. 2009). An epidemical transmission pattern is seen primarily in subtropical and temperate regions (Kono and Kim 1969). In tropical regions JEV usually exhibits an endemic pattern with scattered cases throughout the year, mainly affecting young children who have not yet acquired protective antibodies. In subtropical areas there is a less clear epidemiological pattern with both occasional epidemics and continuous low level transmission. Though primarily affecting children, when JEV emerges into a new territory it may cause disease in a higher proportion of adults as there is no prior immunity (van den Hurk et al. 2009).

JEV has been encountered in new regions during the last 50 years. Migratory birds and wind-enhanced movement of mosquitoes are potential spreading mechanisms (Mackenzie et al. 2006). There are likely many factors behind the spread of JEV to new areas but it is believed to be, at least partly, linked to development of agricultural practices with increased irrigation

(creating breeding sites for mosquitoes) and higher density of farm animals (with pigs serving as virus amplifiers) (Solomon et al. 2000, Keiser et al. 2005).

It is not fully understood how JEV is sustained during the winter in colder climates. It has been suggested that the virus is reintroduced by migratory birds, that it may persist in hibernating mosquitoes or bats, and that vertical transmission may play a part (Mackenzie et al. 2006). The importance of vertical transmission is indicated by the fact that JEV has been isolated from both wild caught male mosquitoes and from larvae reared to adults (Dhanda et al. 1989).

Japanese encephalitis in humans and other animals

Japanese encephalitis in humans

The first isolation of Japanese encephalitis virus was made in 1935 but epidemics of encephalitis are described from the 1870s and forwards in Japan (Rosen 1986, Solomon et al. 2000).

The clinical manifestation of JE can be that of an acute encephalitis syndrome; fever, headache, nausea, motor weakness/deficits, abnormal behaviour, convulsions, seizures and coma (Lowry et al. 1998, Borah et al. 2011). Other patients show symptoms of acute flaccid paralysis or only abnormal behaviour (Gould and Solomon 2008). The majority of infected patients, however, only develop subclinical infections or show mild flu-like symptoms (Solomon et al. 2000). In fact, probably less than 1% of infected people develop clinical disease (Fischer et al. 2010).

The case fatality rate varies, but has in some outbreaks been between 20-40% (Kono and Kim 1969, Parida et al. 2006, Wang et al. 2007). A case-control study by Ding et al (2007) evaluating patients 6-27 years post infection showed that long-term neurological deficits, such as motor defects and seizures, were evident in over 20% of the patients. Solomon et al. (2002) studied the outcome of 144 patients with JEV infections. Their results showed that 23% had severe sequelae and only 19% had made a full recovery at discharge.

There is no effective specific treatment for the disease, and interferon alpha trials, for example, have not proven to be effective (Solomon et al 2003).

Japanese encephalitis in domestic animals

JEV is known to be a reproductive pathogen in pigs but typically does not cause any other clinical signs in adult pigs. Non-immune sows infected before 60-70 days of gestation may abort or have litters containing stillborn, mummified foetuses and weak piglets (Platt and Joo 2006, Givens and Marley 2008). Pigs have been used as sentinels in epidemic areas, as their seroconversion often precedes human cases by 2-3 weeks (Daniels 2002).

A study by Lindahl et al (2012a) in the Mekong Delta of Vietnam showed an association between decreased reproductive performance and JEV seropositivity only in young sows

(<1.5 years). The authors propose that this is because of the endemic disease pattern in the region, leading to early immunity.

Horses can, similarly to humans, develop fatal encephalitis, although the case fatality rate is lower than for other viral encephalitis (e.g. West Nile virus encephalitis). Symptoms include initial fever and ataxia, in some cases aggravating to include blindness, coma and subsequent death (SJV 2008). Other domestic animals, including dogs and sheep, seroconvert after being introduced to the virus but show no signs of disease nor do they appear to produce substantial viremias (Mackenzie et al. 2006, Shimoda et al. 2011).

Virus detection and Japanese encephalitis diagnostics

Virus detection in vectors

Virus detection can be made by different methods. Virus isolation is a slow, expensive process that requires virus to be viable. Thus it has in many instances been replaced by new methods such as the polymerase chain reaction (PCR) (Murphy 1999). PCR is a highly sensitive and quick method that detects viral nucleic acid. By using a pair of primers (forward and reverse) that binds to the 3' ends of each DNA strand, adding a DNA polymerase as well as additionally needed substrates and running it through specific thermal cycles, amplification of a region of the DNA strand is achieved. To detect RNA a RT-PCR is used. A reverse transcriptase (RT) enzyme first produces complementary DNA (cDNA) from the RNA before the PCR. Nested PCR procedures are used to increase the tests sensitivity; this is achieved by using two pairs of primers, with the second pair binding to a region within the product of the first primers (Maclachlan and Dubovi 2010). In semi-nested PCR protocols, for the second PCR reaction, one of the outer primers is used in conjunction with an inner primer (Johansen et al. 2002).

Johansen et al. (2002) investigated different methods for JEV detection in mosquitoes under simulated field conditions. They found that when dead mosquitoes are stored in warm and humid environments the viability and hence the probability for virus isolation quickly decreases and is close to non-existent after one day. The virus RNA however is more stable and can be detected up to 14 days post mortem when mosquitoes are stored in thymol and analysis is made with a RT-PCR or semi-nested RT-PCR. The RT-PCR showed limitations in detecting virus RNA when the size of the mosquito pools tested increased. In contrast the semi-nested RT-PCR was able to detect one JEV infected mosquito in a pool of 1,000 non infected mosquitoes. Pooling of mosquitoes is often needed for practical and economical reasons (Johansen et al. 2002).

One problem encountered when using PCR for detecting virus is the inhibition of the PCR by mosquitoes. Exactly how mosquitoes inhibit the reaction is not fully understood but suggested mechanisms include, but are not limited to, inhibition of enzymes by proteins and lipids (Lanciotti et al. 2000) and inhibition by eye pigment (Nimmo et al. 2006).

A nested PCR protocol established at SLU indicated improved sensitivity for JEV detection in pools of mosquitoes when samples were diluted, likely lessening the inhibition of mosquitoes on virus detection (Lindahl 2012).

The infection rate of mosquitoes is often calculated as the minimum infection rate (MIR). The MIR is calculated as follows:

$$\left(number\ of\ positive\ pools \Big/ total\ number\ of\ mosquitoes\ tested \right) \times 1000$$

This yields an estimate of the minimum number of infected mosquitoes per 1,000 specimens. It does not take into account the possibility of there being more than one positive mosquito per positive pool (CDC 2011).

Diagnostics in animals

Virus detection in blood requires sampling of the animal in a viremic state. Experimental infection of pigs with JEV produces a viremia that generally last for one to three days, being detectable in blood from day two to day five post infection (Williams et al. 2001, Shimizu et al. 1954). As for virus detection in vectors, there are a number of different methods that can be used to detect virus in blood, with different merits and disadvantages.

Detection of antibodies against JEV in pigs is used as a screening method to assess the JE status of an area and to predict outbreaks. Neutralizing antibodies reach a maximum two weeks post infection (Shimizu et al. 1954). Detection of antibodies can be achieved by different methods, the most common being haemagglutination inhibition (HI) and enzyme-linked immunosorbent assays (ELISAs). Cross reaction is a concern as closely related flaviviruses (e.g. West Nile virus, Murrey Valley virus and Dengue virus) may yield false positives (Mackenzie et al. 2006).

Recently dogs have been studied as possible sentinels (Shimoda et al. 2011). One problematic aspect with using pigs as sentinels is the fact that they themselves play a vital part in disease propagation (Daniels 2002). Dogs on the other hand do not seem to produce a significant viremia, thus not contribution to transmission (Mackenzie et al. 2006). Serological screening of dogs may also be more representative when looking at human risk in urban environments (Shimoda et al. 2011).

Diagnostics in humans

Diagnosis of human JEV infection relies most commonly on symptoms in combination with detection of antibodies in cerebrospinal fluid or serum (Solomon et al. 2008). IgM antibodies are usually detectable a couple of days after initial symptoms and may remain so for months. Rising positive titres of IgG antibodies point to an on-going infection, IgG antibodies are however even more prone to cross reactions than IgM (Mackenzie et al. 2006). Commercially available IgM ELISAs are becoming more readily available, but show differences in sensitivity and specificity (Moore et al. 2012).

Vectors

Mosquitoes as vectors

The time it takes for an egg to develop to an adult mosquito varies but can under warm conditions be less than 10 days. Blood meals are a source of nutrients for the production of eggs (though not all females require a blood meal for reproduction). Female mosquitoes can fly several kilometres looking for breeding sites or suitable blood meals, males are thought to be less mobile. Female mosquitoes are believed to locate hosts by different factors including body temperature and CO_2 (Marquardt 2004). The mosquito seeks for an appropriate site to penetrate the skin after having landed on a host. It then searches for intradermal blood (vessel or haemorrhage), a behaviour termed probing. The probing time (i.e. until blood sucking commences) varies between species as well as with other factors.

For a certain mosquito species, to serve as a competent vector of a disease agent, several factors interplay. The vector must exist in sufficient abundance close to potential hosts. The vector must feed on a viremic host, the pathogen must be able to cross the gut epithelium and infect the vector and the vector must finally feed or at least probe on a new host. The time from blood feeding on a viremic host until the vector is able to transmit the infection is the extrinsic incubation period. It is temperature dependent, with shorter periods seen with higher temperatures. For flaviviruses it is usually 10-14 days (Marquardt 2004). In one study the extrinsic incubation period was 20 days at 20°C and as short as 6 days at 28°C (Takahashi 1979 cited by van den Hurk et al. 2009). There are two terms used in this context; vector competence which refers to vector-pathogen interaction (e.g. susceptibility of infection, extrinsic incubation period) and vector capacity which in addition entails other vector attributes (e.g. population density, breeding and blood meal preferences) and interactions between vector and host.

Vector species

In 1938 JEV was isolated from *Culex tritaeniorhynchus* (Mitamura et al. 1938 cited by van den Hurk et al. 2009) and has since been isolated from more than 30 species of mosquitoes (van den Hurk et al. 2009). Today *Cx .tritaeniorhynchus* is established as the most important vector of JEV (Mackenzie et al. 2004). Other members of the *Cx. vishnui* subgroup (to which *Cx. tritaeniorhynchus* belongs) as well as *Cx. gelidus* and *Cx. fuscocephala* are also recognized vectors (Rosen 1986). *Cx. quinquefasciatus* has been shown to be capable of

transmitting JEV experimentally (Banerjee et al. 1977 cited by Rosen 1986) and to be naturally infected (Rosen 1986, Lindahl 2012). Other genera from which JEV has been isolated include *Aedes, Anopheles* and *Mansonia* (Rosen 1986, Thenmozhi et al. 2006, Liu et al. 2012). Likewise, isolates of JEV have been made from *Armigeres* mosquitoes (Liu et al. 2012) and the mosquito has in an experimental setting shown potential as a vector of JEV (Chen et al. 2000). The potential role in the epidemiology of JE for these genera remains to be further investigated.

Risk factors and weather variables

Closeness to pigs and rice fields are established risk factors that increase the risk of contracting JE, and rice fields are favoured breeding sites for *Cx. tritaeniorhynchus* (Keiser et al. 2005, Liu et al. 2010, Impoinvil et al. 2011). Rice field density correlates positively to vector abundance (Richards et al. 2010). In an outbreak of JEV in Australia additional factors believed to contribute was closeness of stagnant water and the density of the human population (Hanna et al. 1996). The total rice production and the areas used for rice cultivation in countries where JE is endemic has been constantly increasing over the past decades (Keiser et al. 2005). Pork production is likewise intensifying in many regions (Cameron 2000, Tisdell 2008).

Vietnam is a wide-stretched country (figure 1). Solomon et al (2000) compared climate differences between southern Vietnam, where the disease pattern of JEV is endemic, and northern Vietnam where JEV occurs in epidemics. There were no notable differences in rainfall but the temperature differed greatly. In the north the number of JE cases peaks during the summer when the temperature rises above 20°C as opposed to the south where both the temperature and the mean number of cases every month is relatively constant (Solomon et al. 2000). A study on weather variables in an urban environment in China showed that temperature and rainfall as well as humidity may have effect on the spread of JEV (Bi et al. 2007). As speculated by the authors, weather variables may influence the transmission by many means, and it is therefore an intricate topic. Possible factors influenced by the weather could be: vector development (e.g. rainfall providing breeding grounds for mosquitoes, higher humidity increasing the mean survival time of mosquitoes) and infection (shortening the extrinsic incubation period) and human behaviour (e.g. people being more inclined to sleep outdoors and wear light clothes in warm temperatures) (Bi et al. 2007). To further demonstrate the complexity, although rain is essential for providing breeding grounds for mosquitoes, excessive amounts may cause flooding and diminish the larval population (Reisen et al. 1976).

The main vectors of JEV, the *Cx. vishnui* subgroup, are known to breed in rice paddies. *Cx. tritaeniorhynchus* is known to breed in varied climates, in northern regions hibernating during winter, but in warmer areas breeding all year around (Reisen et al. 1976). Rice paddies are likewise preferred breeding grounds for *Cx. gelidus* (Abu Hassan et al. 2010) but this species is also known to breed in wastewaters (Whelan et al. 2000). The urban *Cx. quinquefasciatus* often breeds in dirty stagnant waters and drains (Epstein 2001). Larval survival rates may

vary greatly. In one study between 0,3 to over 50% of the larvae survived to become pupae (Sunish et al. 2006). Factors influencing the survival rates are, among others, paddy height, water temperature (increased temperature is positively correlated to larval survival rates) and the chemical properties of the water (e.g. pH, dissolved oxygen, ammonia and nitrates levels) (Sunish and Reuben 2001, Sunish et al. 2006).

In a recent study in India, the population of *Cx. tritaeniorhynchus* was seen to increase during and after the rainy season, this was also the period with the highest MIR. For *Cx. gelidus,* there was no clear change in density associated with seasons but the MIR was similarly highest during and post rain season. Infection rates were also seen to fluctuate depending on the temperature (Upadhyayula et al. 2012).

Though JEV is generally viewed as a rural concern, JEV infection is also seen in humans living in urban environments (Tseng et al. 2003, Bi et al. 2007). Lindahl et al (2012b) investigated JEV vectors in the urban environment of Can Tho city in the Mekong delta region of Vietnam. They found that vectors known to be competent transmitters of JEV were present in urban homes. Pigs in the close vicinity were associated with an increased number of competent vectors, in particular *Cx. tritaeniorhynchus.* An increased density of people in the household was related to higher numbers of *Cx. quinquefasciatus.*

Blood feeding preferences

Though *Cx. tritaeniorhynchus*, like most mosquitoes, is opportunistic, studies on blood feeding habits and preferences have shown it to be mainly zoophagic, preferably feeding on cattle, and to a much lesser degree on other vertebrates such as pigs, humans, birds and goats (given abundance of said hosts) (Reuben et al. 1992, Philip Samuel et al. 2008). In the study by Philip Samuel et al. (2008), *Cx. gelidus* was shown to feed mainly on cattle and pigs and to a lesser extent on humans, ducks and goats, these result are comparable to those previously obtained by Reuben et al. (1992). Other studies have however shown *Cx. tritaeniorhynchus* to have a greater preference for human and pig feeding than previously mentioned studies, which, as speculated by Philip Samuel et al. (2008), might be because of differences in host availability in the different studies, showing the adaptable nature of *Cx. tritaeniorhynchus*.

Cx. quinquefasciatus similarly displays opportunistic patterns with blood meals commonly originating from avian and mammal hosts (including human, dogs and pigs) (Molaei et al. 2007, Garcia-Rejon et al. 2010).

Multiple feeding increases the potential for disease transmission (Kuno and Chang 2005). In the study by Philip Samuel et al. (2008) the percentage of mosquitoes having fed on multiple hosts ranged from 0.1 to 28% (the later for *Cx. tritaeniorhynchus*). It was also shown to be a significant difference in the feeding patterns between different seasons.

Vietnam

JE is an important public health issue in Vietnam. An inactivated JE vaccine, produced nationally, was introduced in 1997, and the immunization initially targeted young children (age one to five years) in high risk areas. The immunization program was expanded and in 2007 included children in 65% of the districts of Vietnam. Statistics from 1999 to 2007 show an average of 2,000 acute encephalitis syndrome cases each year (with a mean annual incidence of 2.4 cases per 100,000 inhabitants), with a decreasing trend over that time period. In districts with extended data JE was found to be the likely cause of about 50% of the acute encephalitis cases. Data from most of the region is lacking however, so the true incidence of JE is difficult to estimate (Yen et al. 2010).

There is a clear trend of rapidly growing economies and intensification of agricultural practices in South Asian countries (Huynh et al. 2006). In Vietnam the volume of pork produced more than doubled between 1996 and 2006. Despite this, swine production is still largely done by small scale production; in 2001 more than 90% of Vietnams pigs came from the household sector with the vast majority of these farms having 10 pigs or less. The Mekong delta region supplied almost 20% of the total Vietnamese pork in 2006 (Tisdell 2008). However rice was the number one commodity produced in Vietnam 2010, making Vietnam the world's 5^{th} largest rice producer (FAO 2012).

Travellers: risk and vaccination recommendations

Available data suggest that the risk for travellers from non-endemic countries to contract JE while visiting endemic countries is low, with less than one out of 1,000,000 travellers affected. There have been 55 published cases where visitors from non-endemic countries contracted JE while visiting endemic areas between 1973 and 2008. Increased risk is seen with increased duration of stay and for people visiting rural areas (Hills et al. 2010).

Vaccination recommendations is usually based on travel itinerary, taking into account factors such as season and planed destination, type of accommodation, duration of stay and activities. Vaccination is recommended for travellers intending to stay over one month in endemic areas and for those who plan to visit rural areas or an area with an on-going outbreak of JE (Fischer et al. 2010).

METHOD AND MATERIAL

The study area

Can Tho city province is the largest province in the Mekong delta region (figure 1) with a population of approx. 1.2 million people in 2011 (SOCTC 2012). Nine districts make up Can Tho city province (figure 1), the two most densely populated being Ninh Kieu with a population density of 8,600 inhabitants per km^2 and Binh Thuy with a population density of 1,639 inhabitants per km^2 (SOCTC 2012), see table 1. Ninh Kieu is further divided into 13 wards and Binh Thuy into eight wards.

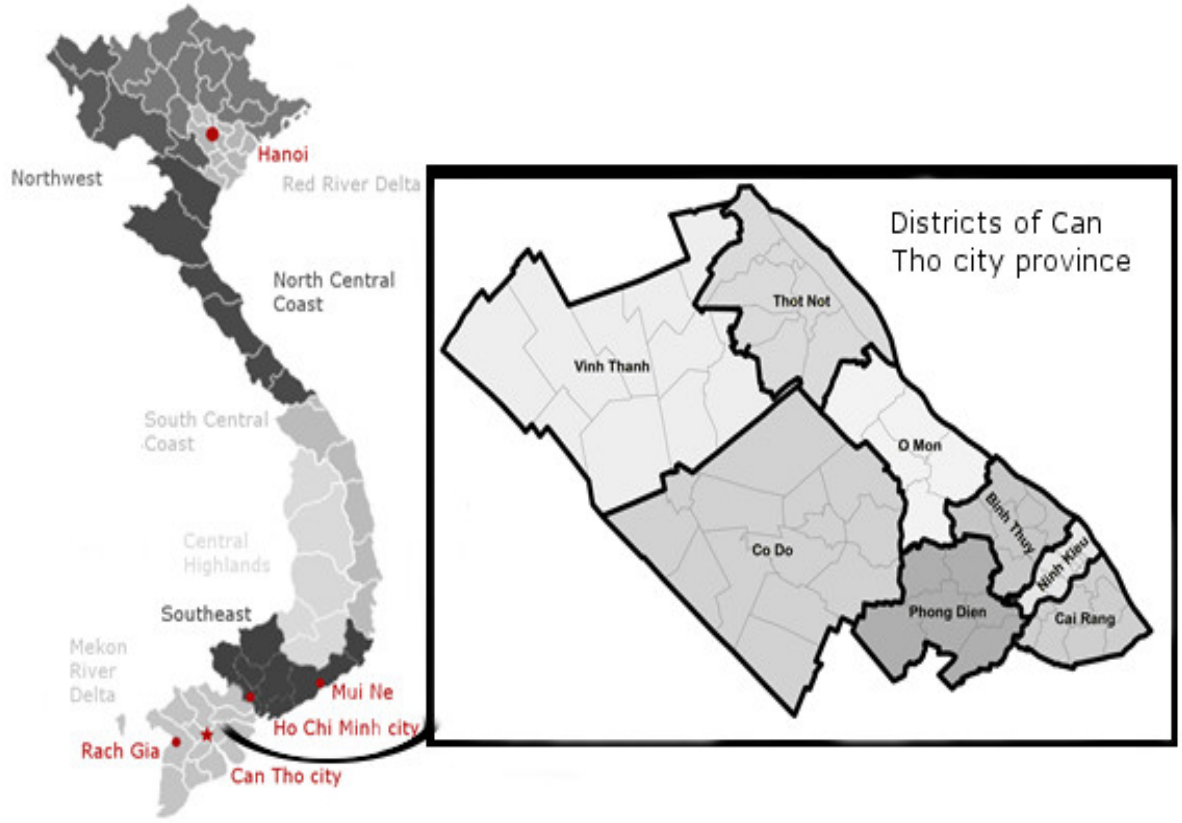

Figure 1. Map of Vietnam and the districts of Can Tho city province (2008); the Co Do district has since been divided into Co Do and Thoi Lai. The study site Can Tho city is marked by a star, the map also shows the two additional locations where questionnaires were handed out, Mui Ne and Rach Gia as well as the capital Hanoi and the largest city, Ho Chi Minh city.

Table 1. Area, average population and population density, 2011 (SOCTC 2012)

	Area (km^2)	Population (inhabitants)	Population density (inhab/km^2)
Can Tho	1,402	1,209,192	863
Ninh Kieu	29	249,451	8,602
Binh Thuy	71	116,349	1,639

The area of land used for agriculture and rice paddies varies greatly between the urban and rural districts. Table 2 shows land use data for the two urban districts in Can Tho where collection of mosquitoes and larvae took place, Ninh Kieu and Binh Thuy, as well as the entire Can Tho city province. The area used for rice paddies all year includes up to three harvests per year (SOCTC 2012).

Table 2. Agricultural land use 2011, Can Tho (SOCTC 2011) (Ha)

	Total land	Land used for agriculture	Land used for rice paddies (all year)
Can Tho	140,895	115,432	91,838
Ninh Kieu	2,926	850	267
Binh Thuy	7,068	3,972	1,502

The climate in Can Tho is tropical, with a dry season from December to April and a rainy season from May to November. Can Tho city experiences increased problems with flooding during the rainy season, the total city area flooded reaching 50% at times (Huong and Pathirana 2011). Average monthly rainfall and temperatures for 2010 and 2011 in the Can Tho city province are displayed in table 3.

Table 3. Average monthly temperature and rainfall for 2010 and 2011, Can Tho city province (SOCT 2011). Data for 2011 is shown in bold

	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
Temp	26.0	27.0	28.4	29.4	30.0	28.1	27.4	27.1	27.6	26.9	27.0	26.4
(C°)	**25.8**	**26.2**	**27.3**	**28.1**	**28.6**	**27.5**	**27.2**	**27.5**	**27.0**	**27.9**	**27.4**	**26.0**
Rainfall	15	0	1	1	67	196	144	215	120	265	204	82
(mm)	**2**	**0**	**104**	**1**	**156**	**181**	**385**	**168**	**152**	**101**	**191**	**55**

Collection sites

In Ninh Kieu, 18 collection sites were chosen and an additional two were located in Binh Thuy (in An Thoi, the ward bordering to Ninh Kieu). Sites in Ninh Kieu were chosen in seven different wards (three in An Khanh, three in An Hoa, three in Xuan Khanh, three in Hung Loi, two in An Phu, two in Cai Khe, one in An Cu and one in Thoi Binh). Figure 3 and 4 show the distribution of collections sites. The selected locations had stagnant waters and were in general situated in housing areas (see table 4 for a description of each collection site and figure 2 shows pictures from some of the sites). Enquiries were made about the presence of pigs in the vicinity of the collection sites.

Table 4. Description of collection sites. NK= Ninh Kieu, BT= Binh Thuy

Site	Area	Description of location	Population density (inhab/km^2) (NKSD 2010)
A	An Khanh (NK)	Water filled ditch, vegetation. Pig farm close. University campus area.	5,374
B	An Phu (NK)	Pool of stagnant water in conjunction with garbage heap. Housing area.	25,333
C	An Phu (NK)	Pool of stagnant water in conjunction with garbage heap/sewage drainage. Housing area/close to market.	25,333
D	An Hoa (NK)	Pool of stagnant water in conjunction with garbage heap/sewage drainage. Housing area/close to market.	16,675
E	An Hoa (NK)	Pool of stagnant water, vegetation. Close to rice field. Housing area.	16,675
F	An Hoa (NK)	Water filled ditch, vegetation. Housing area	16,675
G	Xuan Khanh (NK)	Pool of stagnant water in conjunction with garbage heap/sewage drainage. Housing area/close to market.	14,630
H	An Thoi (BT)	Pool of stagnant water, vegetation. Housing area.	2,051
I	Xuan Khan (NK)	Pool of stagnant water/drainage, vegetation. Close to housing area.	14,630
J	Xuan Khan (NK)	Pool of stagnant water. Housing area.	14,630
K	An Thoi (BT)	Water filled ditch. Close to housing area/industries.	2,051
L	Thoi Binh (NK)	Water filled ditch. Housing area, pigs close.	27,468
M	Hung Loi (NK)	Water filled ditch, vegetation. Housing area.	9,949
N	An Khanh (NK)	Water filled ditch in conjunction with garbage heap, vegetation. Housing area.	5,374
O	Hung Loi (NK)	Water filled ditch, vegetation. Housing area.	9,949
P	Hung Loi (NK)	Water filled ditch, vegetation. Housing area.	9,949
R	An Khanh (NK)	Pool of stagnant water, vegetation. Housing area.	5,374
S	Cai Khe (NK)	Pool of stagnant water. Housing area.	3,754
T	Cai Khe (NK)	Pool of stagnant water in conjunction with garbage heap, vegetation. Housing area.	3,754
U	An Cu (NK)	Water filled ditch, running water in some areas, stagnant in others, vegetation. Housing area.	26,572

Figure 2. Examples of collection sites.

Figure 3 and 4 outlines the pig and population densities for the wards of Ninh Kieu, based on data collected in 2008 for all wards (Lindahl et al 2012b). An Binh has the highest pig density and lowest population density. Maps were created using ESRI ArcMap (software Redlands, California, USA).

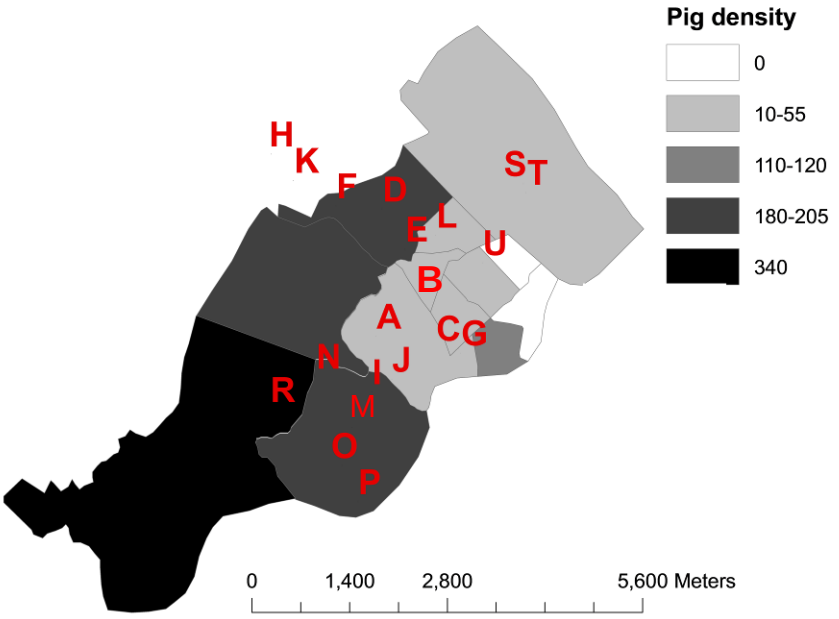

Figure 3. Map showing pig densities (heads/km^2) for the different wards of Ninh Kieu. Collection site locations are marked.

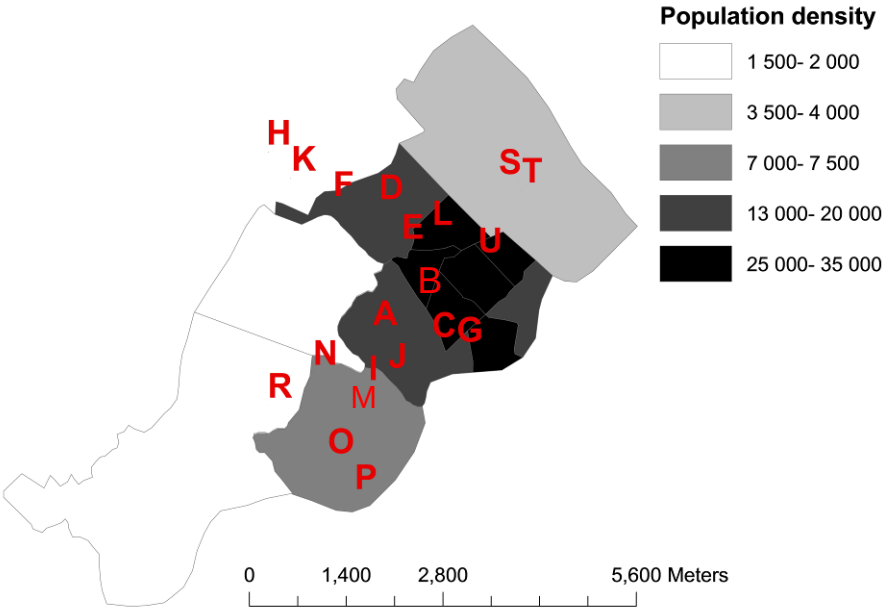

Figure 4. Map showing population densities (inhab/km^2) for the different wards of Ninh Kieu. Collection site locations are marked.

Mosquito and larvae collection

Mosquitoes were collected from the beginning of October until the beginning of November in 2012. Collection took place during 35 minutes at dusk, when JEV vectors are known to be active (Wada and Jojiro. 1970), using two handheld nets. The mosquitoes were killed overnight by freezing and subsequently identified. Mosquitoes were identified according to a key by Reuben et al (1994). Female mosquitoes were identified down to genus and members of the *Culex* genus were further identified down to species. A tentative identification of male mosquitoes was performed based on the key for females. Attempts to separate male mosquitoes of the *Cx. vishnui* subgroup were not made.

Mosquitoes were pooled based on location, sex and species, with a maximum of 67 specimens per tube and then stored in a freezer. TRIzol® Reagent (Applied Biosystems®, California, USA), a phenol and guanidine isothiocyanate mixture, was added, to inactivate virus (Blow et al. 2004, Hofmann et al. 2000) and conserve RNA integrity (Hofmann et al. 2000) before transport to Sweden. Due to time restraint and practical reasons, when the number of mosquitoes from one collection site exceeded 200 specimens, female mosquitoes were separated from males and a random minimum of 200 specimens identified, primarily females. The remaining specimens were counted and sorted as unidentified females and males respectively. Some specimens were not identifiable because of loss of characteristics during pre-identification handling. These are grouped together with specimens that were not identified due to time restraint. All female mosquitoes, plus the male mosquitoes from 10 collection sites, were transported to the National Veterinary Institute, Sweden, for PCR analysis.

Larvae were collected from the pools of water using small plastic containers in association with mosquito collection at the same site. They were killed by ethanol and then stored in a freezer until transport to Sweden before which Trizol was added. Larvae identified as belonging to the *Culex* genus, according to a key by Rattanarithikul (1982), were separated from unidentifiable/others. When the number of collected larvae exceeded 100 specimens, a random selection of 100 specimens were identified and the remaining specimens sorted as unidentified.

Coordinates for the collection sites were obtained using a Garmin eTrex® H handheld GPS.

PCR analysis

Mosquitoes and larvae were analysed using a nested RT-PCR (Lindahl et al. unpublished data). Some pools containing a small number of specimens were grouped together to obtain an adequate amount of test material.

RNA extraction

For each pool to be tested, 250 µl of homogenized sample was added to 750 µl of Trizol reagent and incubated for five minutes at room temperature. 200 µl of chloroform was added to the samples which were then shaken for 15 seconds and subsequently incubated for two to

three minutes at room temperature. Samples were then centrifuged at 12,000 rmp for 15 min in a refrigerated microcentrifuge. The aqueous phase was mixed with 500 µl of isopropanol and incubated over night at -20°C. After removing the isopropanol the remaining pellet (containing RNA) was washed with 75% ethanol, then air dried. Finally 20 µl RNAse free water was added and the samples stored at -70°C until further use. The mosquito samples were diluted 1:10 and 1:100 with RNAse free water.

Nested RT-PCR

The AgPath-ID™ One-Step RT-PCR Kit was used, as it minimizes sample handling and thus contamination risk, followed by a quatitative PCR (qPCR), using the Path-ID™qPCR Master Mix Protocol (TaqMan® probe-based real-time PCR), to enhance sensitivity. In all assays negative and positive controls were included. In the RT-PCR the forward primer 5'-TGGATGACSACKGARGAYATG-3' (emf1) and the reverse primer 5'-GGGTCTCCTCTAACCTCTAG-3' (vd8) were used (Pierre et al. 1994). For the qPCR the inner forward primer 5'-ATCTGGTGYGGYAGTCTCA-3' and inner reverse primer 5'-CGCGTAGATGTTCTCAGCCC-3' were used (Pyke et al. 2004). In the qPCR the dual labelled probe 5'-FAM-CGGAACGCGAWCCAGGGCAA-TAMRA-3' was used (Pyke et al. 2004).

From the prepared samples, 2 µl of extracted and diluted RNA (1:10 and 1:100) was added to 23 µl of the RT-PCR master mix (Applied Biosystems®/Ambion, California, USA), containing 12.5 µl 2X RT-PCR buffer, 1 µl 25X RT-PCR enzyme mix, 8.7 µl nuclease-free water and primers to a final concentration of 160 nM. The enzyme mix included ArrayScript™ Reverse Transcriptase and AmpliTaq Gold® DNA Polymerase.

The reverse transcription (RT) and amplification was run in a Biometra T3000 Thermocycler. RT was accomplished by 45 minutes at 45°C, followed by denaturation for 10 minutes at 95°C. Subsequently PCR was carried out by 15 seconds of denaturation at 95°C followed by elongation at 60°C for 60 seconds, repeated for 50 cycles.

For the nested PCR, 1 µl of the RT-PCR product was added to 24 µl of Path-ID™qPCR Master Mix (Applied Biosystems®, California, USA) (12.5 µl 2X RT-PCR buffer, 1 µl probe, 8.5 µl nuclease-free water), and primers to a final concentration of 400 nM, giving a final volume of 25 µl. The nested PCR was run and analysed using a Corbett Rotorgene RG-3000, starting with denaturation at 95°C for 10 minutes, followed by 40 repeated cycles consisting of 15 seconds at 95°C and 60 seconds at 60°C.

Survey

In addition to the epidemiological study, a small survey was carried out. Two separate questionnaires were used; one targeting native Vietnamese people and the other tourists. Both questionnaires had questions regarding age, sex, occupation, vaccination status,

measurements taken to avoid mosquito bites and awareness of JE. The questionnaire targeting Vietnamese people also had questions concerning risk factors such as ownership of pigs and closeness to rice fields. For the tourists additional questions addressed duration of stay, reason for visiting Vietnam and type of accommodation during stay.

The questionnaires aimed at Vietnamese people were handed out in Can Tho city and in Mui Ne. Tourist questionnaires were in addition to the previously mentioned areas also handed out in Rach Gia. Descriptive statistics were used to evaluate most of the results. In addition, Chi2 and Fisher's exact test were used to evaluate the correlation between age and vaccination status, comparing three age groups (<30 year, 30-49 years and ≥50 years). PASW Statistics 18, SPSS Inc, was used to perform the analysis.

RESULTS

Mosquitoes

A total of 4,110 mosquitoes were collected. Male mosquitoes constituted 70.4% and female mosquitoes 29.6% of the total number of specimens caught. Mosquitoes from eight different genera (*Culex, Mansonia, Aedes, Anopheles, Mimomyia, Uranotaenia, Armigeres* and *Luzia*) were identified. Only mosquitoes of the *Culex* genus were identified to species and consisted of *Cx. quinquefasciatus, Cx. tritaeniorhynchus, Cx. pseudovishnui* and *Cx. gelidus*. For female mosquitoes the most common species found was *Cx. quinquefasciatus* (70.8%) followed by *Cx. tritaeniorhynchus* (26.3%) and *Cx. gelidus* (3.9%), excluding unidentified specimens. Similarly, for male mosquitoes *Cx. quinquefasciatus* was the most abundant species identified (88.5%) followed by mosquitoes from the *Cx. vishnui* subgroup (7%) and *Cx. gelidus* (0.8%). One collection site, "O" in the Hung Loi ward, yielded 45.6% of all mosquitoes caught. When data from site "O" is excluded *Cx. quinquefasciatus* represents 57.4 % of the female mosquitoes and *Cx. tritaeniorhynchus* 33.5%.

The number of specimens caught in a specific site ranged from 1-1874 specimens, with an average of 205.5 (median 108). At the sites "O", "G" and "S" the number of mosquitoes caught exceeded what was practically possible to identify, hence a minimum of 200 specimens were identified (primarily females). In general some specimens were not possible to identify due to damages/lack of characteristics acquired during pre-identification handling. These were also classified as unidentified. Table 7 outlines the results for the different sites.

Larvae

A total number of 368 larvae were collected (table 5). Larvae were found at nine of the 20 sites. At one site, "M", the number of larvae exceeded 100 specimens and due to time constraints 100 random larvae were identified, the remainders were counted. Larvae from the *Culex* genus were separated from others and represented 95.7% of all identified larvae. Three sites yielded 87% of all larvae, site "M", "O" and "S". At all three sites an above average number of mosquitoes were also collected.

Table 5. Mosquito larvae found at the different sites, Can Tho, Vietnam, October-November 2012

Collection site	B	C	D	F	K	N	O	S	T	**Total**
Larvae	22	11	3	6	2	167	92	61	4	**368**
Culex	14	10	-	6	1	100	92	61	4	**288**
Other/unidentified	8	1	3	-	1	67	-	-	-	**80**

RT-PCR results

For the mosquitoes, a total of 13 pools out of 130 tested were positive. The total number of mosquitoes tested was 3,353 (only males from 10 of the 20 sites were tested, males constituted 63.7% of the tested specimens). Of the positive pools, seven were pools of male mosquitoes and six were pools of female mosquitoes. Collection site "P" had four positive pools, collection site "N" had three positive pools, collection site "J" and "M" both had two positive pools and "O" and "K" each had one positive pool. Unidentified mosquito pools made up four of the positive pools, three were pools of *Cx. quinquefasciatus*, two were from mosquitoes identified as belonging to the *Cx. vishnui* subgroup, and two consisted of *Anopheles*. Samples of *Aedes* and *Armigeres* each contributed with one positive pool. No larvae pools were positive. See table 6 for more information on the positive pools and figure 5 for the geographical distribution. These PCR results have not been confirmed by another PCR protocol nor through DNA sequencing.

Table 6. Positive pools in RT-PCR

Site	**Genus/species**	**Sex**	**Number of specimens in pool**
J	*Anopheles*	Female	1
J	*Aedes*	Female	1
K	*Armigeres*	Female	1
M	*Unidentified*	Male	21
M	*Cx. quinquefasciatus*	Male	56
N	*Cx. quinquefasciatus*	Male	35
N	*Cx. vishnui subgroup*	Male	10
N	*Unidentified*	Male	3
O	*Unidentified*	Male	50
P	*Cx. quinquefasciatus*	Female	10
P	*Unidentified*	Female	3
P	*Anopheles*	Female	2
P	*Cx. vishnui subgroup*	Male	6

Table 7. Number of mosquitoes collected at the different sites (Can Tho, Vietnam, October-November 2012)

Collection site	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**R**	**S**	**T**	**U**	**Total**
Female mosquitoes	20	40	63	11	8	118	72	1	33	24	34	18	77	60	440	34	27	47	42	46	**1216**
Cx. quinq.	-	31	54	9	3	41	57	-	17	19	11	7	52	14	200	10	5	40	19	33	**622**
Cx. tritaen.	11	-	2	1	2	58	11	1	14	2	14	11	11	31	16	19	13	3	19	7	**246**
Cx. gelidus	3	-	-	-	-	7	1	-	2	-	4	-	3	9	3	-	3	-	1	-	**36**
Cx. pseudov.	-	-	-	-	-	-	-	-	-	-	-	1	-	1	1	-	-	-	-	-	**3**
Mansonia	4	-	-	-	-	1	-	-	-	-		-	-	-	-	-	3	-	-	-	**8**
Aedes	-	-	1	-	-	2	-	-	-	1	1	-	-	-	-	-	-	-	-	-	**5**
Anopheles	-	2	-	-	-	1	-	-	-	1	2	-	1	1	-	2	1	-	-	-	**11**
Uranotaenia	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**2**
Luzia	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	**1**
Armigeres	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	**1**
Unidentified	2	7	6	1	2	7	3	-	-	1	-	-	10	4	220	3	2	4	3	6	**281**
Male mosquitoes	8	95	63	8	2	96	331	-	106	43	33	-	136	50	1434	25	30	280	80	60	**2892**
Cx. quinq.	2	82	57	7	1	52	230	-	96	42	12	7	110	35	-	16	6	157	61	58	**1030**
Cx. vishnui spp	3	1	-	-	-	14	1	-	4	-	18	6	3	100	-	6	16	-	4	-	**81**
Cx. gelidus	-	-	-	-	-	1	-	-	6	-	-	1	-	2	-	-	-	-	-	-	**9**
Mimomyia	-	-	-	-	-	23	-	-	1	-	-	-	-	-	-	-	-	-	-	-	**24**
Mansonia	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	6	-	-	-	**8**
Aedes	2	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	**3**
Armigeres	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	**1**
Uranotaenia	-	-	-	-	1	5	-	-	-	-	-	-	2	-	-	-	-	-	-	-	**8**
Unidentified	-	12	6	1	-	1	100	-	4	1	2	-	21	-	1434	2	2	125	15	2	**1730**
Total mosquitoes	**28**	**135**	**126**	**19**	**10**	**214**	**403**	**1**	**144**	**67**	**67**	**26**	**213**	**110**	**1874**	**59**	**57**	**329**	**122**	**106**	**4110**

Figure 5. Distribution of RT-PCR positive mosquito pools in the Ninh Kieu district. Each red mark represents a site which had one or more positive pools.

Survey results

A total number of 83 questionnaires were handed out and answered. Of those, 58 were answered by Vietnamese people and 24 by tourists/foreigners.

Tourist/foreigner survey results

The age ranged from 20 to 63 years, with an average of 40 years and a median of 36.5 years. The sex distribution was 41.7% females (n=10) and 58.3% (n=14) males. The nationalities of those who participated were Australian (n=5), British (n=3), Canadian (n=1), Dutch (n=2), French (n=3), German (n=2), Malaysian (n=1), New Zealander (n=1), Norwegian (n=1), Spanish (n=1), Swedish (n=3) and Swiss (n=1). The duration of stay in Vietnam ranged from 12 days to 12 years, though only one person stated that they would stay longer than four weeks. 41.7% (n=10) stated that they had heard about JE but only 8.3% (n=2) answered that they were vaccinated against JEV. A majority 75% (n=18) took some precaution against mosquito bites, the most common being mosquito repellent, 94.4% (n= 17), followed by mosquito net 11.1% (n=2) and other precautions 11.1% (n=2). No one stated that they used long clothing to protect against mosquitoes and 16.7% (n=3) used more than one type of prophylaxis. Table 8 outlines the use of prophylaxis for both Vietnamese people and tourists. There was one (4.2%) person who answered that they studied/worked in Vietnam while 22 (95.7%) responded that they were in Vietnam on vacation.

Table 8. Number of people using different mosquito prophylaxes. Vietnamese and tourist/foreigners

	No precaution	Repellant	Mosquito net	Long clothing	Other
Vietnamese	4	35	23	12	9
Tourist/foreigners	2	17	2	0	2
Total	6	52	25	12	11

Vietnamese survey results

The age ranged from 14-50 years with an average of 34 years and a median of 28 years. The sex distribution was 48.3 % females (n=28st) and 51.7 % males (n=30). A majority, 79.3% (n=46) stated that they had heard about JE and 44.8% (n=26) answered that they were vaccinated against JEV. There was no significant difference between males and females. Most participants, 93.1% (n=54) answered that they took some precaution to avoid mosquito bites. The most common being mosquito repellant, 64.8% (n= 35) followed by mosquito net, 42.6% (n=23), long clothing, 22.2% (n=12), and 16.7% (n=9) stated that they took some other precautions against mosquitoes. Out of those taking precautions against mosquitoes 29.6% (n=16) responded that they used more than one type of prophylaxis. Only one (1.7%) person stated that they owned pigs and 23 (39.7 %) that they lived close to stagnant water. Participants under the age of 30 were significantly more likely to respond that they were vaccinated compared to other age groups, in the age range 14-29 years 62.5% stated that they were vaccinated (table 9).

Table 8. Vaccination status for different age groups (Vietnamese people). Both older age categories had significantly lower proportion vaccinated than people under 30 ($p < 0.05$)

	<30 years	30-49 years	≥50 years	Total n
Vaccinated n (% for age group)	20 (62.5)	5 (31.25)	1 (11.1)	26
Not vaccinated n (% for age group)	12 (37.5)	11 (68.75)	8 (88.89)	31
Total n (%)	32 (100)	16 (100)	9 (100)	57

DISCUSSION

This study focused on the presence of JEV vectors at potential breeding grounds. *Cx. quinquefasciatus* is known to prefer urban habitats and breed in stagnant waters such as sewers and other polluted waters (Epstein 2001), explaining its abundance in the present study. The two sites known to have pig farms in the close vicinity instead showed a higher

proportion of *Cx. tritaeniorhynchus*, which is in accordance with previous observations in the area (Lindahl et al. 2012b). It is however not possible to say whether some of the other sites had pigs close, as information about pigs was obtained solely by asking people in the neighborhood, and may not be correct. Nor was it possible to get hold of accurate and current statistics on pig density for the different wards (the data displayed in figure 3 is from 2008). Sites which displayed a higher than average (for this study) proportion of *Cx. tritaeniorhynchus* had vegetation and often water filled ditches. The study by Lindahl et al. (2012b) had a higher proportion of *Cx. gelidus* (25%) than what was seen in the present study (3.9% of female and 0.8% of male mosquitoes) possibly indicating that the type of waters included in this study is not this species' preferred breeding sites. Mosquitoes from other genera than *Culex* were only sporadically found. It is often challenging to distinguish members of the *Cx. vishnui* subgroup (Reuben 1994) and the possibility of misidentification, especially within that group, must be considered. As males were identified using a key developed for females, separation of *Cx. vishnui* members was not attempted.

In this study, there was a large proportion of males collected, which may be explained by the fact that the collections took place around presumed breeding sites, compared to collections made close to potential hosts where females tend to dominate. Male mosquitoes are also thought to be less mobile than female, staying close to breeding grounds, were oviposition likely occurs, waiting for female mosquitoes (Marquardt 2004).

Larvae were only found at nine of the 20 collection sites. It is possible that there in some instances existed more beneficial/preferred sites in the vicinity. At some sites it was not possible to get access to the entire range of the waters and larvae might therefor have been present in another area. Some waters may have had chemical and physical properties making them unsuitable as breeding grounds. For example, Paaijmans et al. (2008) suggest that soil particles can raise the temperature in waters during midday, driving larvae which prefer to be close to the surface (such as *Culex*) to migrate deeper, disturbing larvae development. Larvae growth and survival rates may also be negatively affected by soil particles because of interference with larvae feeding (Ye-Ebiyo et al. 2003). Collection took place at the end of the rainy season, with the occasional heavy rainfalls and flooding, possibly affecting the larvae population. The main part of the collected larvae came from three sites. They were all sites at which an above average (for this study) number of mosquitoes were caught. There were however other sites with large quantities of mosquitoes where no larvae were found. The vast majority of larvae identified (>95%) belonged to the *Culex* genus, suggesting that the types of sites targeted in this study may indeed be of importance in the urban epidemiology of JEV.

The results of the RT-PCR were 13 positive pools out of 130 tested. The PCR results need to be interpreted cautiously as they have not been confirmed with another method (either another PCR protocol or DNA sequencing). Using a nested PCR does increase sensitivity but also the risk of false-positive results because of increased risk of contamination (Maclachlan and Dubovi 2010). Another consideration is that when samples have low amounts of RNA, positive results are not always reproducible. Detection can be influenced by factors in the samples, such as background RNA, inhibiting the RT reaction or the PCR, having a greater impact on samples with small amounts of template (Levesque-Sergerie et al. 2007). It has also

been suggested that non-reproducibility of results from samples with small amounts of template can be because of random factors influencing whether or not each template is amplified, termed the “Monte Carlo” effect. This is proposed to occur when templates are diluted beyond a certain level (Karrer et al. 1995).

When looking at the distribution of positive pools, all but one were in relative vicinity of each other. Seven pools originated from collections made in the Hung Loi ward and five from neighboring wards. Hung Loi has an, for Ninh Kieu, average population and pig density (see figure 3, 4 and 5). The 13 positive pools originated from six different sites. The likelihood of detecting JEV in male mosquitoes is substantially smaller than in female mosquitoes. In this study seven of the 13 positive pools were male mosquitoes. Male mosquitoes did however represent 63.7% of the specimens tested. Pools of *Cx. quinquefasciatus* represented three of the positive pools. JEV has previously been detected in *Cx. quinquefasciatus,* in Can Tho city (Lindahl 2012). To this author’s knowledge, natural vertical transmission has not previously been shown for *Cx. quinquefasciatus.*

The *Cx. vishnui* subgroup includes some of the most important JEV vectors (*Cx. tritaeniorhynchus, Cx. pseudovishnui, Cx. vishnui*) and two of the positive pools contained males from this subgroup. Of the positive pools, four contained female mosquitoes identified as *Aedes, Anopheles* or *Armigeres.* All four pools consisted of only one to two specimens, which, combined with the fact that JEV is not commonly detected in those species, renders a guarded interpretation of those positive results. JEV may be detected in a mosquito either because the specimen is in fact infected, or because it has recently fed on a viremic animal. In the latter case the mosquito has infected blood in the gut but may not necessarily become infected, and is not necessarily a competent vector able to transmit the virus.

The collection of material for this study took place during a relatively brief period of time resulting in a limited number of specimens. Each site was only visited once, thus variations in factors such as weather variables may have a substantial influence over collected data. To further explore the urban epidemiology of JEV and the properties of breeding sites for JEV vectors, it would be of value to collect material during a longer time period. Collecting from the same sites on more than one occasion, would minimize the effects of temporary weather variables. Other means of capture (such as overnight traps) might yield a larger number of specimens, if practically possible. It would be desirable to collect a larger number of larvae and identify them to species level. In conjunction with (if possible) water analysis, such information would give a better understanding of which potential JEV vectors breed at which type of site. Knowledge of vector breeding sites and preferences can give indications on possible intervening points in the JEV cycle by vector control and pinpoint risk areas.

Survey

The scope of the survey was limited to 83 respondents. It was however clear that both native Vietnamese people as well as tourists, in general take some precaution to avoid mosquito bites. Only two tourists stated that they were vaccinated against JEV. As all but one tourist

planned to stay less than four weeks in Vietnam, vaccination is not usually recommended (Fischer et al. 2010).

Of the Vietnamese people a majority (79.3%) answered that they had heard about JEV but only 44.8% stated that they were vaccinated. An immunization program was introduced in 1997 in Vietnam, initially targeting young children in high risk districts in the northern region. It has since expanded and included 65% of the districts in Vietnam in 2007 (Yen et al. 2010). This explains the finding that people under the age of 30 years were significantly more likely to be vaccinated compared to other age groups. It is also possible that not all who were vaccinated as children are aware of it.

CONCLUSIONS

This study confirms previous reports about the presence of known, competent JEV vectors in the urban parts of Can Tho city, Vietnam. The proportional abundance of *Culex* larvae and mosquitoes, in particular males, found at the selected collection sites indicates that the targeted sites (i.e. stagnant waters) function as vector breeding grounds. As such they may play a vital part in the urban epidemiology of JE. Knowledge of vector ecology and preferences is of great importance when designing vector control strategies and assessing the potential for disease propagation. Therefor, further investigations of urban breeding grounds are warranted.

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