

Japanese Encephalitis Virus in Pigs and Vectors in the Mekong Delta

With Special Reference to Urban Farming

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Abstract

Japanese encephalitis Virus (JEV) is a mosquito-borne flavivirus in East and South Asia, estimated to cause 60 000 human cases of Japanese encephalitis each year. The main transmission cycle for JEV is via mosquito vectors, mainly *Culex* species, between pigs and wading birds, the reservoir hosts. Incidental infection in humans can result in encephalitis with 30% case fatalities, and half of the survivors may have neurological sequelae. Some of the most important vectors, such as *Culex tritaeniorhynchus*, commonly breed in rice fields, and the disease is therefore mainly considered to be a risk in rural areas. However, increasing urbanisation creates needs and opportunities for urban agriculture, and since pork is popular in Southeast Asia, it is common to keep pigs in cities. With this in mind, this thesis focuses on three important aspects of JEV circulation; the pig, the vectors and the virus itself.

Pigs are usually only clinically affected by JEV during pregnancy. Sows in the area around Can Tho city in the Mekong delta region of South Vietnam, where JEV occurs endemically, were investigated for the association between JEV seropositivity and reproduction. In total 315 sows were included, with 60% seropositive. In sows less than 1.5 years of age, seropositivity was associated with the occurrence of more stillborn piglets, but this association could not be observed when all sows were included in the analysis.

Mosquitoes were collected in households within and surrounding Can Tho city. The numbers of the zoophilic vectors *Cx. tritaeniorhynchus* and *Culex gelidus* were positively associated with pigs, whereas *Culex quinquefasciatus*, an anthropophilic vector, was positively associated with the number of people in the household. Of the 7885 mosquitoes collected, seven mosquito pools, collected close to pigs, were positive for JEV using nested RT-PCR. Four PCR fragments were similar to JEV genotype III and three to genotype I, indicating that both genotypes circulate in Can Tho city at the same time. Within Can Tho city all pigs sampled (43/43) were seropositive for JEV.

These findings demonstrate that JEV can be a public health issue in urban as well as in rural areas and that it is associated with few reproductive problems in sows in an endemic area.

Keywords: Arbovirus, flavivirus, emerging infectious disease, mosquitoes, zoonosis, vector-borne disease, urban agriculture

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Dedication

To Hanna (2002-2009), and every child that never was allowed to grow up due to the incurable diseases and the injustices in this world. Remember it doesn't matter if you fall off an angel horse when you are an angel, because you will land on the clouds.

There are more things between heaven and earth, Horatio, than are dreamt of in our imagination. (Act I, Scene V)

And thus conscience does make cowards of us all; And thus the native hue of resolution is sicklied o'er by the pale cast of thought. (Act III, Scene I)

William Shakespeare. Hamlet

Following primary infection of a host with certain viruses, a lifelong relationship can be established.

Asha Mathur *et al.* (1989). Evidence for latency of Japanese encephalitis virus in T-lymphocytes. *Journal of General Virology* 70(2)

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Lindahl, J, Boqvist, S, Ståhl, K, Thu, HTV, Magnusson, U (2012).
Reproductive performance in sows in relation to Japanese Encephalitis Virus seropositivity in an endemic area. *Tropical Animal Health and Production* 44(2), 239–245.
- II Lindahl, J, Chirico, J, Boqvist, S, Thu, HTV, Magnusson, U (2012).
Occurrence of Japanese Encephalitis Virus mosquito vectors in relation to urban pig holdings. *American Journal of Tropical Medicine and Hygiene* accepted for publication. Doi:10.4269/ajtmh.2012.12-0315.
- III Lindahl, J, Ståhl, K, Chirico, J, Boqvist, S, Thu, HTV, Magnusson, U.
Circulation of Japanese Encephalitis Virus in pigs and mosquito vectors within Can Tho city, Vietnam (submitted manuscript).

Papers I-II are reproduced with the permission of the publishers.

Abbreviations

<i>Ae.</i>	<i>Aedes</i>
AES	Acute encephalitis syndrome
Arbovirus	Arthropod-borne virus
Bp	Base pair
C	Capsid protein
ct	cycle treshold
<i>Cx.</i>	<i>Culex</i>
E	Envelope protein
ELISA	Enzyme-linked immunosorbent assay
HI	Haemagglutinin inhibition
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
MIR	Minimum infection rate
MLE	Maximum likelihood estimate
NS	Non-structural protein
OD	Optical density
PCR	Polymerase chain reaction
prM	pre-membrane protein
qPCR	quantitative or real-time PCR
RNAi	RNA interference
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
UTR	Untranslated region

1 Introduction

Japanese encephalitis virus (JEV) is a zoonotic flavivirus capable of infecting most vertebrates, although clinical disease seems to occur mainly in humans, pigs and horses. In humans, it is the cause of Japanese encephalitis (JE), a dreaded emerging zoonotic disease in large parts of Asia (Mackenzie *et al.*, 2004; Saxena *et al.*, 2011). In spite of the existence of vaccines, JE is a leading cause of childhood encephalitis in Asia (Halstead & Jacobson, 2003) and more than 60 000 human cases are estimated to occur every year in the affected areas (Campbell *et al.*, 2011). Japanese encephalitis virus can be an economically important reproductive pathogen in pigs (Platt & Joo, 2006) and causes fatal encephalitis in horses (Calisher & Walton, 1996). The virus is transmitted by mosquitoes; it is an arthropod-borne virus, an arbovirus, amplified by pigs and wading birds, whereas humans and other mammals are accidental, dead-end hosts (Rosen, 1986; Peiris *et al.*, 1992; Erlanger *et al.*, 2009).

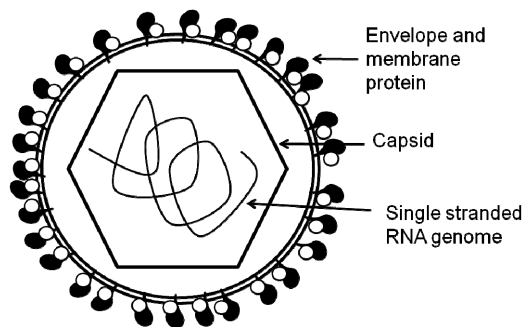
The main incriminated vector, *Culex tritaeniorhynchus*, commonly breeds in rice fields, where wading birds are often present, and thus JE has mainly been considered of importance in rural areas (Rosen, 1986; Endy & Nisalak, 2002; Halstead & Jacobson, 2003). In a world with more than seven billion people (Bloom, 2011) almost half of the population lives in areas where JEV is transmitted (Erlanger *et al.*, 2009). The proportion of people living in cities recently exceeded 50% (Satterthwaite *et al.*, 2010; Bloom, 2011) and in Southeast and East Asia seven million of these urban inhabitants are involved in urban agriculture (van Veenhuizen & Danso, 2007), in which pigs are common (Schiere & van der Hoek, 2001). With these global changes in focus, the overall goal of this thesis is to increase the knowledge about JEV in the Mekong Delta region of Vietnam and to provide evidence of the presence of JEV circulation and exposure in urban areas.

1.1 Japanese encephalitis virus

Japanese encephalitis virus belongs to the viral family Flaviviridae which consists of three genera; pestiviruses, hepaciviruses and flaviviruses (MacLachlan & Dubovi, 2011; Unni *et al.*, 2011), the latter also called arboviruses group B.

1.1.1 Description of the virus

Flaviviruses are small, enveloped viruses, around 50 nm, with a single-stranded positive-sensed RNA genome of approximately 11 000 nucleotides (Figure 1) (Unni *et al.*, 2011). Three genes encode the structural proteins, the capsid protein (C), the envelope protein (E), and the pre-membrane protein (prM), which is spliced into the membrane protein (Kaufmann & Rossmann, 2011). The seven non-structural proteins (NS) are important for virus replication with RNA-polymerase activity mainly performed by the NS5 protein (Hurrelbrink & McMinn, 2003; Kim *et al.*, 2007).



5'UTR C prM E NS1 NS2A NS2B NS3 NS4A NS4B NS5 3'UTR

Figure 1. Japanese encephalitis virus schematic representation of the virus particle and the genome structure. UTR: untranslated region. Modified from Unni *et al.* (2011).

The mutation rate is generally high in RNA viruses, which may explain their high potential for causing emerging diseases (Holmes, 2004; Cleaveland *et al.*, 2007), but vector-borne RNA viruses have a lower degree of mutation (Jenkins *et al.*, 2002). Genetic drift is the main evolutionary mechanism for JEV (Halstead & Jacobson, 2003) although re-combinations can also occur (Twiddy & Holmes, 2003; Holmes, 2004).

Based on the nucleotide sequences of the prM and E genes, which are the most variable (Uchil & Satchidanandam, 2001), JEV has been divided into five genotypes. Genotype V is the most genetically divergent (Mohammed *et al.*, 2011), followed by genotype IV, whereas the other three genotypes are the

most similar and wide-spread (Figure 2) (Solomon *et al.*, 2003; Pan *et al.*, 2011).

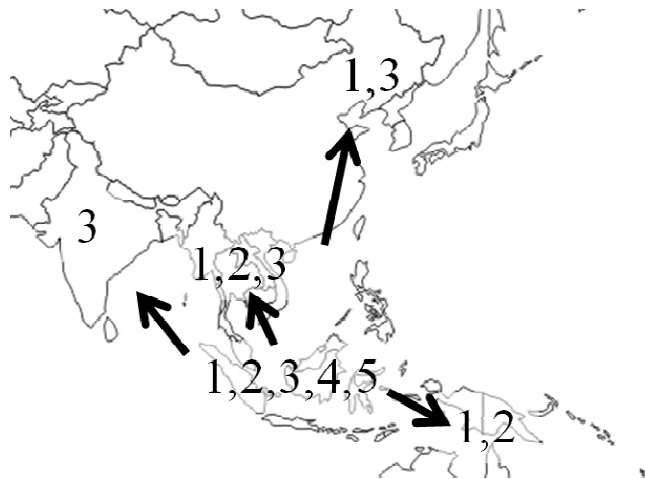


Figure 2. Proposed spread of JEV genotypes in Asia. Modified from Solomon *et al.* (2003)

1.1.2 Virus detection

Traditionally, detection of JEV has been done by virus isolation, which is considered the gold standard for arboviruses, but has drawbacks compared to molecular methods (Black & Salman, 2005; Hall *et al.*, 2012). It is labour-intensive, time-consuming, and requires that the virus remains viable (Lanciotti *et al.*, 2000; Black & Salman, 2005). In dead mosquitoes it is difficult to isolate virus after only one day (Johansen *et al.*, 2002).

Polymerase chain reaction (PCR) is a sensitive molecular method for detecting DNA. To detect viral RNA a reverse transcriptase PCR (RT-PCR) is used, which in the reverse transcriptase (RT) step first transforms RNA into DNA.

Detection of virus in live vertebrates is usually possible only during the viraemia, which is short and limited to two to three days in pigs (Williams *et al.*, 2001), or from cerebrospinal fluids in encephalitic cases (WHO, 2007). Both RT-PCR and virus isolation can be used to detect flavivirus in vectors. For economic and practical reasons mosquitoes are often analyzed in pools of mosquitoes grouped together (Black & Salman, 2005).

1.1.3 Serological response and detection

The flaviviruses are divided into serological groups, or antigenic complexes, based on cross-reactions in serological testing (Figure 3). In the JE group there are a number of other mosquito-borne viruses causing encephalitic syndromes,

such as West Nile virus, St Louis encephalitis virus, Murray Valley encephalitis virus, Kunjin virus, and Usutu virus (De Madrid & Porterfield, 1974; Calisher *et al.*, 1989). In serological tests, JEV antibodies are more likely to cross-react with these viruses than with flaviviruses from other serological groups, such as Dengue virus, Yellow fever virus and Tick-borne encephalitis virus. Japanese encephalitis virus is considered to consist of only one serotype, although there might be some antigenic variations between strains (Mackenzie *et al.*, 2006). In individuals immune against one flavivirus, infection with another flavivirus induces a broad spectrum of antibodies which may cross-react with even more flaviviruses (Makino *et al.*, 1994; Bosco-Lauth *et al.*, 2011) and viraemia does not always occur (Williams *et al.*, 2001).

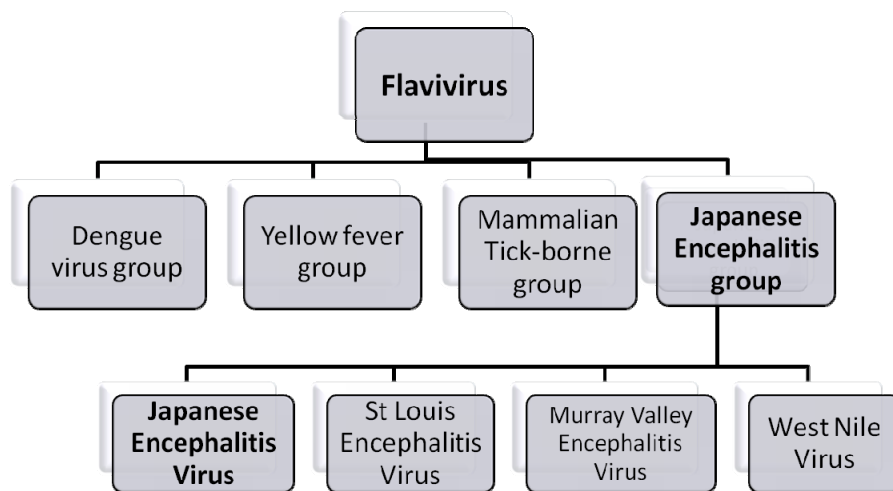


Figure 3. Schematic description showing four of the serological groups in the flavivirus genus, and four of the viruses in the Japanese encephalitis virus serological group.

In primarily infected pigs, IgM antibodies increase two to three days post infection and peaks after one week (Burke *et al.*, 1985a). IgG antibodies start increasing one week post infection (Ishii *et al.*, 1968; Kuno, 2001). If a sow is immune, maternal antibodies, 80% of which being IgG, will be transferred via the colostrums to the newborn piglets (Redman, 1979). This passive immunity is usually lost well before six month of age (Scherer *et al.*, 1959d; Sota *et al.*, 1991).

All serological tests for flaviviruses are more or less prone to cross-reactions. Both IgG and IgM antibodies can be detected with a Haemagglutinin

inhibition test (HI), which is a relatively cheap method, but also discriminates poorly between different flaviviruses, especially between viruses of the JEV serological group and the Dengue group (Grossman *et al.*, 1973; De Madrid & Porterfield, 1974; Kimura-Kuroda & Yasui, 1986; Kuno, 2003).

Enzyme linked immunosorbent assays (ELISA) may detect either IgM or IgG, and are semi-quantitative, since the optical density (OD) values obtained are often correlated to antibody titres (Robinson *et al.*, 2010). Indirect IgG ELISAs for JEV in pigs have been shown to have a good correlation with HI results and also to be discriminating against other common viral swine pathogens (Xinglin *et al.*, 2005; Yang *et al.*, 2006). IgM ELISA may cross-react to a lesser degree than IgG ELISA (Makino *et al.*, 1994; Koraka *et al.*, 2002; Kuno, 2003; Robinson *et al.*, 2010) and is often used to detect recent infections.

1.2 The epidemiology of Japanese encephalitis

Japanese encephalitis virus is maintained in a cycle where pigs, birds and possibly also bats, are infected by the primary culicine vectors (Figure 4) (Mackenzie *et al.*, 2004; Pan *et al.*, 2011). These reservoir hosts are essential for the maintenance of JEV as they amplify the virus, without marked clinical signs. Ideal reservoir hosts have a rapid reproduction and a high turn-over, which continually supplies new susceptible individuals (Kuno & Chang, 2005).

For a zoonotic vector-borne disease, bridge vectors which feed on both humans and animals are also necessary (Schmidt & Ostfeld, 2001). Many mosquito species are generalized, opportunistic feeders (Garrett-Jones, 1964) and can therefore serve as bridge vectors between the reservoir hosts and incidental hosts, such as humans and horses (Gubler, 1996). These incidental hosts usually do not develop high viraemia and are thus considered dead-end hosts in the epidemiology of JEV. However, these accidental infections can have serious health consequences for the infected individuals.

The large geographic area in which JEV is present comprises both temperate and tropical regions. In its northern range, JE outbreaks occur in late summer when vectors are active; further south the transmission periods are longer, and in the tropical South and Southeast Asia endemic transmission occurs all year, but may be affected by monsoon rains (Halstead & Jacobson, 2003). In early studies of the epidemiology of JEV in Japan, Buescher & Scherer (1959) described the seasonal pattern. Piglets were usually born in April, during the pre-emergence period. The number of *Cx. tritaeniorhynchus* increased with a peak in June, followed by a peak in the number of infected mosquitoes. The first detection of JEV in mosquitoes usually occurred some

weeks before the first detection in pigs, and after the peak in vector abundance, high numbers of susceptible pigs and birds were infected.

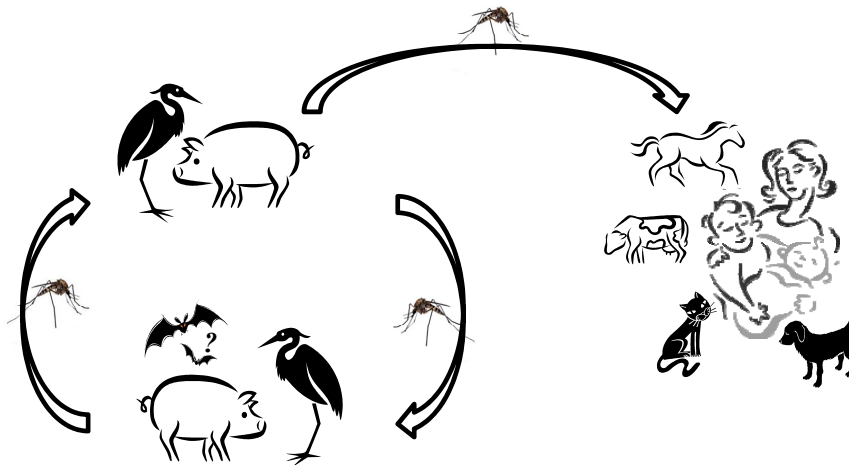


Figure 4. The transmission cycle of Japanese encephalitis virus between its amplifying hosts, with occasional transmission to novel vertebrates by oligophilic bridge vectors.

In temperate regions JEV must either survive in its vectors or in its vertebrate hosts, or be reintroduced every season (Rosen, 1986). In Japan it has been shown that the virus seem to be both continually reintroduced and surviving locally (Nabeshima *et al.*, 2009).

1.3 Infection in vertebrates

1.3.1 Japanese encephalitis infection in pigs

Pigs are important in the epidemiology of JEV since they amplify the virus and are often kept close to humans. Viraemia occurs two to five days after experimental infection and usually lasts for one to three days (Williams *et al.*, 2001) although it has been possible to isolate JEV longer in single cases (Nitatpattana *et al.*, 2011).

In non-pregnant female pigs, infection with JEV is usually asymptomatic. In pregnant sows JEV may cause reproductive “SMEDI” symptoms, in even Stillbirth, Mummification, Embryonic Death and Infertility, and piglets born with neurological signs, while the sows may show only mild fever and inappetence (Burns, 1950; Hosoya *et al.*, 1950; Shimizu *et al.*, 1954). Foetuses are individually infected and some may be healthy, whereas others die. After ten weeks the foetuses develop immunocompetence and can resist infections

(Salmon, 1984; Gresham, 2003). The common reproductive symptoms make JEV infection in pigs difficult to distinguish clinically from infections by other viruses, such as porcine parvovirus, Aujeszky's disease (pseudorabies) virus, classical swine fever virus or porcine reproductive and respiratory syndrome virus (Platt & Joo, 2006; Daniel Givens & Marley, 2008).

Following JEV infection, boars may have transient reduced libido, orchitis, and abnormal spermatozoa. The virus can be venereally transmitted, and exported semen may thus pose a risk for introducing JEV into a country (Maes *et al.*, 2008). In young piglets, JEV can cause wasting syndromes and non-suppurative meningoencephalitis, (Yamada *et al.*, 2004; Yamada *et al.*, 2009).

1.3.2 Japanese encephalitis infection in birds and bats

Based on studies demonstrating the presence of viraemia and high prevalence of antibodies, wading and ardeid birds, such as egrets and herons, have traditionally been considered the main reservoir of JEV in nature (Buescher, 1956; Scherer *et al.*, 1959b), but there may be other birds equally important as reservoir hosts (Buescher *et al.*, 1959; Rosen, 1986). Indeed, in one study only few JEV vectors had fed on ardeid birds (Reuben *et al.*, 1992). The importance of domestic poultry in the epidemiology is still not clear, but it is known that they may develop viraemia and seroconvert (Miyamoto & Nakamura, 1969; Johnsen *et al.*, 1974; Rosen, 1986).

In a similar manner to birds, bats have been proposed as important reservoirs for JEV and could transfer virus long distances. Different species of bats have been shown to be naturally infected with JEV, and they may be the explanation to how virus overwinters in temperate regions (Kuno, 2001; Mackenzie *et al.*, 2008) since JEV also has been isolated during winter months (Miura *et al.*, 1970; Sulkin *et al.*, 1970; Cui *et al.*, 2008; van den Hurk *et al.*, 2009).

1.3.3 Japanese encephalitis in humans

Japanese encephalitis is a serious disease in humans and usually around 30% of clinical cases are fatal, although case fatality rates have been reported between four and 60% in outbreaks (Igarashi, 2002; Halstead & Jacobson, 2003; Bista & Shrestha, 2005; Yen *et al.*, 2010). The spectrum of clinical signs is wide, ranging from mild fever to all the symptoms of a meningoencephalomyelitis (Lowry *et al.*, 1998; Solomon *et al.*, 2000; Halstead & Jacobson, 2003). In addition to the fatalities, JEV cause disabilities which may be further poverty-promoting. More than 50% of surviving patients show neurologic sequelae, such as behavioural changes, paralyses or seizures, and some may remain severely disabled (Solomon *et al.*, 2000; Halstead & Jacobson, 2003; Maha *et*

al., 2009). Infections with JEV are, however, subclinical in most cases, with up to 1000 subclinical infections for every clinical case (Southam, 1956). In regions where JEV has recently been introduced, disease usually affects all age groups, in contrast to endemic regions where almost all adults are immune, and clinical cases occur mainly in children (Halstead & Jacobson, 2003).

Although JEV has been isolated from aborted foetuses (Chaturvedi *et al.*, 1980), little is known about the importance of JEV on human reproduction. A high proportion of women of childbearing age are expected to be immune in endemic areas, and thus the effects of infections during pregnancies may be observed first when the virus is introduced into new regions (Tsai, 2006).

1.3.4 Japanese encephalitis virus in other animals

Japanese encephalitis virus may cause a fatal neurotropic infection in horses, similar to the disease in humans. Clinically the disease is similar to other equine encephalitides, and it is often subclinical, or causes a mild fever (Gould *et al.*, 1964; Calisher & Walton, 1996).

In experimental infections young mice are sensitive to JEV, and, as in pigs, the foetuses are infected in pregnant animals (Fujisaki *et al.*, 1977; Mathur *et al.*, 1986). Infection in the last third of pregnancy can result in pups born with antibodies (Mathur *et al.*, 1981). Wild mice seem to be largely genetically resistant to JEV (Brinton & Perelygin, 2003) and rodents are considered unimportant for the epidemiology of the disease (Scherer *et al.*, 1959a). Ruminants, dogs and cats seroconvert without clinical signs (Ilkal *et al.*, 1988; Shimoda *et al.*, 2010; Shimoda *et al.*, 2011).

1.4 Vectors

Arboviruses are transmitted by biological vectors, with the virus infecting and replicating in the vector (Higgs & Beaty, 2005). The flaviviruses can be subdivided into four groups according to their vectors: viruses infecting only mosquitoes, viruses infecting vertebrates with no known vector, tick-borne, and mosquito-borne viruses (Gaunt *et al.*, 2001; Cook & Holmes, 2006; Calzolari *et al.*, 2012). Moreover, the mosquito-borne viruses are subdivided into viruses causing hemorrhagic fevers, such as Dengue virus and Yellow fever virus which are transmitted mainly by *Aedes* species, and neurotropic viruses, including JEV, which are transmitted mainly by *Culex* species. (Gaunt *et al.*, 2001; Gould, 2002).

1.4.1 Vector competence and vectorial capacity

Vector competence is an estimate of the proportion of mosquitoes given an infected meal that become infected and able to transmit the virus. All mosquito species are not competent to transmit all viruses and the vector competence may even differ between geographic subpopulations of a species (Kramer & Ebel, 2003). The vectorial capacity is a measure of the number of potentially infective bites that an individual will be exposed to during one day from one vector species (Black & Moore, 2005). Apart from vector competence, other important factors for the vectorial capacity are the probability that a vector feeds on a specific host, which is dependent on both the feeding frequency and the proportion of meals from that specific host, the vector density in relation to the host density, and vector survival (Macdonald, 1956; Saul *et al.*, 1990; Kramer & Ebel, 2003; Black & Moore, 2005).

Host preferences for the blood meal vary between species and geographic subpopulations. However, most mosquitoes are, in fact, opportunistic and oligophilic with host preferences dependent on the densities of vertebrate hosts (Garrett-Jones, 1964; Kuno & Chang, 2005). Disrupted feeding may result in multiple feedings on different hosts which increase the risk of disease transmission (Klowden & Zwiebel, 2005).

1.4.2 Virus in the vectors

Infection of a mosquito starts with feeding on a viraemic host. After the extrinsic incubation period, in even, the temperature-dependent time until the mosquito is infectious after ingesting virus (Takahashi, 1976; Saul *et al.*, 1990), the mosquitoes secrete virus in the saliva while searching, probing, for blood in the host. Probing is sufficient to transmit virus (Muangman *et al.*, 1972) and consequently prolonged probing increase the risk of pathogen transmission (Klowden & Zwiebel, 2005).

Several barriers in the mosquito may prevent it from becoming a vector for a virus. There is a mesenteric dose-dependent infection barrier and therefore the proportion of mosquitoes infected depends largely on the extent of the host's viraemia (Hardy *et al.*, 1983; Leake, 1992; Mellor, 2000). A mosquito with a poor vector competence can thus become infected if the original blood meal has a sufficiently high viral titre (Gresser *et al.*, 1958; Takahashi, 1976).

Although mosquitoes lack an adaptive immune response they are not defenceless against viral infections (Barillas-Mury *et al.*, 2005). RNA interference (RNAi) helps the mosquito to break down viral mRNA, and may constitute a barrier for viral release into the haemocoel (Keene *et al.*, 2004; Sanchez-Vargas *et al.*, 2004), from which virus subsequently can infect the salivary glands (Leake & Johnson, 1987).

Breeding and vertical infection

The larval breeding sites for mosquitoes are aquatic, and some species are very specific in their habitat requirements, whereas others may exploit a wide range of breeding sites. The abundance of mosquito larvae and their development may depend on a number of factors in the water, such as water depth, salinity, fertilizers, and the presence of predatory fish and insects in the water (Sunish & Reuben, 2001; Sunish & Reuben, 2002). The larval survival rate can vary between 0.3 and 50% (Sunish *et al.*, 2006). Even though rain may provide more breeding grounds and more mosquitoes, other mosquito species may benefit from dry periods (Vythilingam *et al.*, 1997; Chapman *et al.*, 2000).

Japanese encephalitis virus has been isolated from adult males and from adults reared from field-caught larvae, providing evidence of vertical transmission and indicating its importance in the epidemiology of JEV (Dhanda *et al.*, 1989). Vertical transmission has been proposed to be one way in which virus may survive in temperate regions (Rosen, 1989; Kramer & Ebel, 2003).

1.4.3 Japanese encephalitis virus vectors

Many species within the family Culicidae (mosquitoes) have been shown to be able to transfer JEV, and the virus has been isolated from more than 25 species (Leake, 1992).

The Culex sitiens group

The most important vectors for JEV are found within the *Culex sitiens* group (Figure 5). In the *Culex vishnui* subgroup, three species are known to be of major importance; *Cx. tritaeniorhynchus*, *Culex pseudovishnui* and *Culex vishnui* (syn. *Culex annulus*). These mosquitoes are zoophilic, feeding on cattle and pigs (Colless, 1959; Mitchell *et al.*, 1973; Reuben *et al.*, 1992; Bhattacharyya *et al.*, 1994; Arunachalam *et al.*, 2004) depending on their availability, and only some percent feed on humans. *Culex tritaeniorhynchus* is a highly competent vector for JEV (Gresser *et al.*, 1958) and commonly referred to as the most important JEV vector (Mackenzie *et al.*, 2004).

Culex annulirostris, in the *Culex sitiens* subgroup, is considered to be the most important vector in the JE outbreaks in Oceania (Kramer & Ebel, 2003), and may locally feed up to 80% on feral pigs (Hall-Mendelin *et al.*, 2012).

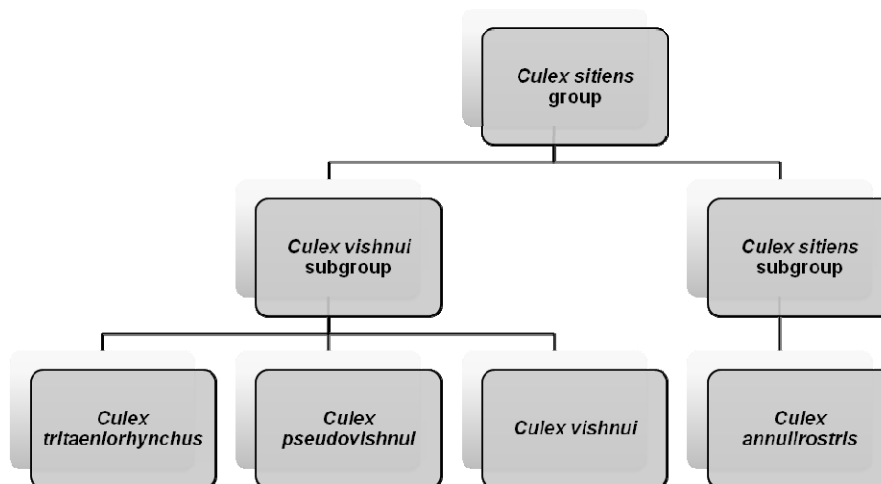


Figure 5. The Japanese encephalitis virus vectors in the *Culex sitiens* group of mosquitoes.

The Culex pipiens complex

The *Culex pipiens* complex includes amongst others *Culex pipiens* (*Culex pipiens pipiens*), the Northern house mosquito, in temperate regions and *Culex quinquefasciatus* (*Culex pipiens quinquefasciatus*, syn. *Culex fatigans*), the Southern house mosquito, in tropical or subtropical regions (Sirivanakarn, 1976; Miller *et al.*, 1996; Fonseca *et al.*, 2004). In areas where the two subspecies overlap, hybrid populations occur and it is difficult to separate them morphologically. *Culex pipiens molestus* is an autogenous subspecies, which does not require a blood meal for ovarian development (Fonseca *et al.*, 2004), and is also a competent vector for JEV, although the transmission rate is lower than for *Cx. tritaeniorhynchus* (Turell *et al.*, 2006; Olsen *et al.*, 2010).

Culex quinquefasciatus is highly anthropophilic, with up to 50-76% feeding on humans (Reuben *et al.*, 1992; Zinser *et al.*, 2004; Hasegawa *et al.*, 2008).

Other important Culex vectors

The highly competent vector *Culex gelidus* is zoophilic, with the majority of blood feeds being on cattle (Colless, 1959; Reuben *et al.*, 1992). It is an invasive species in Northern Australia, with JEV subsequently being isolated from it there (Muller *et al.*, 2001; van den Hurk *et al.*, 2001). *Culex fuscocephala* is an important vector in parts of Asia and is equally as competent a vector as *Cx. tritaeniorhynchus* in laboratory experiments (Muangman *et al.*, 1972), with the same preferences for cattle and pigs

(Colless, 1959; Reuben *et al.*, 1992; Bhattacharyya *et al.*, 1994). *Culex bitaeniorhynchus* may feed on birds, humans and pigs, and less on cattle (Reuben *et al.*, 1992).

Other Culicidae vectors

Aedes aegypti and *Aedes albopictus* are important vectors for Dengue and Yellow fever virus, feeding on humans to a large extent (Gaunt *et al.*, 2001; Ponlawat & Harrington, 2005). Both are competent vectors for JEV in laboratories and may transfer the virus vertically (Rosen *et al.*, 1985; Rosen, 1987). They breed in all sorts of natural and artificial containers, such as car tyres and flower pots (Knudsen, 1995; Strickman *et al.*, 2000). In addition, *Ae. albopictus* is an invasive species which has dispersed throughout the world (Knudsen, 1995; Gratz, 2004; Hubalek, 2008).

Japanese encephalitis virus has also been isolated from Anopheles species and Mansonia species, and the latter genus has been suggested to be able to harbour JEV during winters (Rosen, 1986; Arunachalam *et al.*, 2004).

1.5 Japanese encephalitis – now and then

1.5.1 History and spread of Japanese encephalitis

In spite of the name, JEV does not originate from Japan. Based on genotyping and phylogenetic studies, it has been suggested that JEV originates from Indonesia or Malaysia (Figure 2) (Le Flohic & Gonzalez, 2011). Epidemics of “summer encephalitis” in humans have been known since the 1870s in Japan, and the causative virus was discovered after the large outbreaks in the 1920s (Rosen, 1986). The disease was first named Japanese B encephalitis, to distinguish it from another, similar disease, the “type A” encephalitis, later named von Economo’s encephalitis lethargica (Rosen, 1986; Dale *et al.*, 2004), but the B has subsequently been dropped from the name.

Japanese encephalitis virus is currently spread over a large part of Asia and Northern Australia (Figure 6). Many countries have adapted vaccination programs which have significantly lowered the burden of morbidity in humans. China had more than one million human cases between 1965 and 1975, and accounted for a large part of the world’s cases before vaccination started (Halstead & Jacobson, 2003). Socioeconomic improvements have decreased JE incidence in many countries, while other countries have experienced increases over the same time, in some instances associated with increased areas of irrigated rice production (Umenai, 1985; Balasegaram & Chandramohan, 2008; Erlanger *et al.*, 2009; Sarkar *et al.*, 2012).



Figure 6. Geographic extent of Japanese encephalitis. Modified from Hills *et al.* (2011).

1.5.2 Potential for future emergence of Japanese encephalitis

The definition of emerging infectious diseases in humans or animals is often vague, but is generally based on the presence of either a new disease agent, or the infection of a recognized agent in a new host species, or the spread into new regions (Daszak *et al.*, 2000). Japanese encephalitis may be considered an emerging infectious disease due to its continuous encroachment into new areas (Mackenzie *et al.*, 2002; Erlanger *et al.*, 2009).

In tropical regions, proportionally more infectious diseases are transmitted by insects than in temperate regions (Wolfe *et al.*, 2007). These tropical vector-borne diseases often fall upon the poor, and the lifelong sequelae are further poverty-promoting (LaBeaud, 2008). Although arboviruses can be transmitted by a wide range of arthropods, mosquitoes (Diptera: Culicidae) are the most important from a veterinary and medical point of view (Eldridge, 2005). Anthropogenic changes are among the most important catalysts for emergence of arboviruses, such as increased travelling and transport of animals and goods, deforestation, altered land use, increased irrigation and creation of water reservoirs, and urbanization (Morens *et al.*, 2004; Gould *et al.*, 2006; Cleaveland *et al.*, 2007). A warmer climate may increase the distribution of certain vectors and prolong the transmission seasons (Russell, 1998; Githeko *et al.*, 2000; Patz *et al.*, 2005). Changes in temperature can also cause mosquito species that today are considered to be insignificant vectors to become more important in the future (Mellor & Leake, 2000).

Thus far, JEV has only been emerging in geographically neighbouring areas but the potential pre-requisites for spread of JEV exist in both Europe and North America (Davison *et al.*, 2003; Gould *et al.*, 2006; Nett *et al.*, 2008). However, it is unlikely that JEV would be identified immediately following an introduction. Zoonotic arboviruses, such as JEV, can circulate among animals for a long time before accidental transmission, “spill-over”, into the human population are noted (Lundström, 1999). In fact, JEV viral RNA has recently been demonstrated in Italian birds and mosquitoes in the *Culex pipiens* complex (Platonov *et al.*, 2012; Ravanini *et al.*, 2012).

Urbanization and urban animal farming

Increasing establishment of anthropophilic vectors, coupled with extensive urbanization in tropical regions, is suggested to be the most important factor in the future for arbovirus emergence (Saxena *et al.*, 2011). Urbanization affects the transmission of diseases in many ways, and ecosystems around urban areas are also affected. The increased temperature in and around cities, “urban heat islands”, is beneficial for many vectors and decreases the effects of seasonal variation (Shochat *et al.*, 2006). Decreased biodiversity provides fewer alternative hosts for vectors and increases the risk of accidental transmission to humans (Schmidt & Ostfeld, 2001; Bradley & Altizer, 2007; Keesing *et al.*, 2010).

Urbanization creates needs and opportunities for urban food production, especially with growing demands for animal products (Yeung, 1988; Rae, 1998; Schiere & van der Hoek, 2001; van Veenhuizen & Danso, 2007). Urban agriculture can be defined as the agriculture occurring within a city or a town (Mougeot, 2000), but the borders may be defined differently, making it difficult to compare results between studies. Urban agriculture is often focused on high value crop and animal products that give a high economical turnover from a limited area, and the scavenging behaviour of many animals, such as pigs, make them suitable to keep without own land (Schiere & van der Hoek, 2001; van Veenhuizen & Danso, 2007). Pigs are also suitable in urban backyard production, since they require little space, reproduce quickly, and provide the possibility to re-use household wastes as feed.

1.5.3 Control and prevention

Control of vector-borne diseases can be done either by reducing the number of vectors, reducing the number of reservoirs, or protecting individuals from becoming infected. In some countries, increased living standards have led to decreased JE incidence without vaccination (Tsai, 1997; Petersen & Marfin, 2005).

Vector control

It is difficult to eradicate a vector species, due to negative environmental effects and high costs (Black & Moore, 2005), but numbers may be significantly reduced using environmental and chemical control measures (Hardy *et al.*, 1983). Vector control programs also need to be maintained, since discontinuation can cause diseases to re-emerge (Petersen & Marfin, 2005).

Mosquito larval populations are density-dependent, which may affect the population's response to control measures. In larval habitats with limited food, larvicidal measurements may actually increase the number of emerging adults (Black & Moore, 2005). Intermittent flooding is an environmentally-friendly and effective way to reduce the number of vectors (Keiser *et al.*, 2005), but limited to use by farmers where there is a well-developed irrigation system (Okuno *et al.*, 1975). Fish are often kept to reduce the numbers of mosquitoes, since some fish species, such as the mosquito fish, predate on mosquito larvae. However, if there are enough larval habitats available, mosquitoes can identify ponds containing this species and avoid them (Angelon & Petranka, 2002). Other fish species prefer to feed on other aquatic predators, and may thus actually increase the number of surviving mosquito larvae (Sunish *et al.*, 2006). Another approach to reduce the number of mosquitoes bred is to minimize larval habitats, which can be achieved by removing artificial containers and providing information campaigns about the importance of keeping lids on water barrels.

Bed nets decrease the number and activity of indoor mosquitoes, and the risk for JE, only if impregnated with insecticides (Dapeng *et al.*, 1994a; Dapeng *et al.*, 1994b). Insecticide-treated nets can be affixed to protect either pigs or humans, both of which reduce the infection rate in humans, although a combination is the most effective (Dutta *et al.*, 2011).

Reservoir control

Wild birds and bats, as well as feral pigs, are hard to control, whereas it is possible to influence pig keeping. Industrialized pig production has been associated with lower JE incidence in humans in Japan, where the decreasing JE incidence has been correlated with the decreasing number of pig farms, in spite of an increase in the total number of pigs (Oya & Kurane, 2007). After the outbreaks at Badu Island, Australia, pig production was centralized outside the main community (van den Hurk *et al.*, 2001; van den Hurk *et al.*, 2008). Although the number of infected mosquito pools decreased in the community afterwards, there were still JEV-positive mosquitoes, and *Cx. annulirostris*, the main vector, changed its behavior to feeding more on humans. The main conclusion was that the relocation of the pigs did not completely eliminate the

risk for humans. The same observation was made in Singapore where concentrating the pig production outside the city decreased the JE incidence, but infections still occurred (Ting *et al.*, 2004).

Vaccines

Vaccination in pigs can be used to prevent stillbirths and yield more piglets born alive per sow (Kawakubo *et al.*, 1969; Wu *et al.*, 1989), being regularly used for this purpose in Korea, Taiwan and Japan (Umenai, 1985; Hsu *et al.*, 2008). However, the high turn-over in the pig population makes pig vaccination inefficient for preventing JE in humans (Grossman *et al.*, 1974; Igarashi, 2002). Vaccination in humans is suggested to be the only long-term measure for reducing human JE incidence but there is still a need for better, safer and cheaper vaccines to reach all people living at risk for JE (Mackenzie *et al.*, 2004; Marfin & Gubler, 2005; Oya & Kurane, 2007). Inactivated vaccine based on the attenuated JEV strain SA14-14-2 is currently the most readily available worldwide. There is also ongoing research with recombinant and DNA vaccines (Dutta *et al.*, 2010).

1.5.4 Japanese encephalitis in Vietnam

The first case of JE in Vietnam was identified in 1964, in the same decade as cases were discovered in Thailand and Cambodia (Igarashi & Takagi, 1992) and Vietnam is currently considered a medium-high risk country for JE (Campbell *et al.*, 2011). The human population exceeds 85 million with 20 million below 15 years of age. Clinical JE cases occurs sporadically in humans, most often in children below 15 years of age, while the proportion of immune adults may approach 100 % (Erlanger *et al.*, 2009).

The number of human cases is estimated to be 1000 to 3000 per year. In addition to a growing population, increases in the rice-growing area and pig production are likely to contribute to disease emergence (Erlanger *et al.*, 2009) Pork is the most common meat source in most Asian countries (Rae, 1998) and is popular in Vietnam, where the increasing production is very important for the country's economy. The rice cultivating area in Vietnam increased by 21 % between 1990 and 2005, at the same time as the swine production increased by 147 %, with more than half of this production taking place in the delta regions, the Red River delta in the North and the Mekong delta in the South (Figure 7) (Erlanger *et al.*, 2009; Vo-Tong *et al.*, 1995). Here, the JE incidence in humans is also the highest (Nguyen & Nguyen, 1995; Yen *et al.*, 2010).

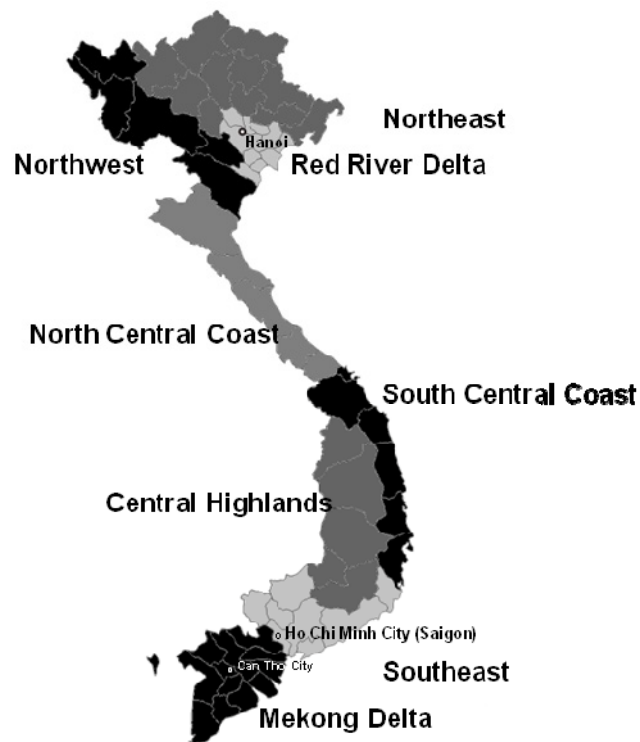


Figure 7. Map of the different regions in Vietnam. The locations of the capital, Hanoi; the largest city, Ho Chi Minh City; as well as Can Tho city are marked.

Although there are a growing proportion of large pig farms, the majority are family farms with one or two sows (Hai & Nguyen, 1997). The farming system in the Mekong delta is often a combination of gardening, aquaculture, livestock and rice fields (Vo-Tong *et al.*, 1995; Kamakawa *et al.*, 2002). Almost half of all rural Vietnamese households keeps pigs, which is the livestock that generates the highest income (Maltsoğlu & Rapsomanikis, 2005). The importance of pigs in Vietnamese agriculture and the suitability of this species for urban farming make it popular in Vietnamese urban animal farming.

In Vietnam, as in many low-income countries, not all suspected human JE cases are confirmed in laboratories, and clinical cases of acute encephalitis syndrome (AES) are reported instead. Yen *et al.* (2010) showed that on average 50 % of the AES cases in Vietnam were caused by JEV. Despite the fact that the number of reported AES cases has been decreasing nationally, some provinces have reported an increase, with AES incidence rates of more than six cases per 100 000 inhabitants (Yen *et al.*, 2010). In 1997 Vietnam

started distributing a domestically-produced JE vaccine to children between one and five years old in 12 high risk districts, and in 2007 the vaccination campaign comprised 65% of the districts (Yen *et al.*, 2010).

In Northern Vietnam the climate is temperate with four distinct seasons, while the climate in the south is tropical with a rainy season and a dry season. In South Vietnam, JE is endemic and cases occur all year around, although peaks can be seen in June-July and February-March (Do *et al.*, 1994; Tan *et al.*, 2010; Yen *et al.*, 2010). In Northern Vietnam, outbreaks occur during the summer months and the difference in epidemic pattern between Northern and Southern Vietnam is more likely to be correlated to temperature than to rainfall (Solomon *et al.*, 2000). Isolates from northern Vietnam in 1986-1989 clustered with isolates from genotype III, whereas isolates from 2001-2002 belonged to genotype I (Nga *et al.*, 2004). The same shift from genotype III to I over time has been seen in other parts of East and Southeast Asia as well (Ma *et al.*, 2003; Morita, 2009; Yun *et al.*, 2010).

2 Aims of this thesis

This thesis focuses on three of the main aspects of the transmission cycle of JEV: pigs as an important reservoir and amplifying host close to humans, the mosquitoes that transmit the virus, and JEV itself. The overall aim was to provide new insights into the epidemiology of JEV in an endemic area, and especially, in an urban environment. The more specific aims were to:

- Assess the seroprevalence for JEV in pigs in and around Can Tho city, Vietnam.
- Evaluate the association between seropositivity for JEV and reproductive performance in pigs.
- Describe the urban animal farming in Can Tho city.
- Record and determine the potential vector species within Can Tho city.
- Evaluate the association between pig keeping and the number of mosquito vectors at urban households, including JEV-infected mosquitoes.
- Establish a sensitive method for detection of JEV RNA in mosquitoes, and circumvent the well-known inhibition caused by mosquitoes.
- Estimate the JEV infection rate in mosquitoes in Can Tho city.

3 Methodological considerations

The materials and methods used are described in paper I-III and are commented upon here.

3.1 Selection of the study area and period

Can Tho city province is located in the Mekong delta in Southern Vietnam (Figure 7). The province is divided into eight districts, which are further subdivided in wards (municipalities) (Figure 8). The Ninh Kieu district corresponds to the urban area of Can Tho city, the “capital of the Mekong delta”, and these names are used synonymously here.

Paper I used material collected by Boqvist *et al.* (2002) in 1999 from four pig farms in districts surrounding Can Tho city. The selection of wards to be included in paper II and III was based on census data from 2008 on human population and animal farming (Anh, 2009). Paper II included households within Ninh Kieu district, where wards were selected with the following pig to human ratios: high (115-213 pigs/1000 people), medium (8-25 pigs/1000 people) and low (0-2 pigs per 1000 people). In paper III, households in Ninh Kieu district and Co Do district were included in addition to the households in paper II. After the wards had been selected, households with and without pigs were invited to participate in the study (Table 1) and were compensated economically per pig sampled and for each trap-night. This convenience sample included households with a range both in the number of pigs and people in the households. Although a randomized selection of households would have been desirable and would have allowed for more generalized conclusions, there was no reason to suspect a bias in the inclusion of participating households.

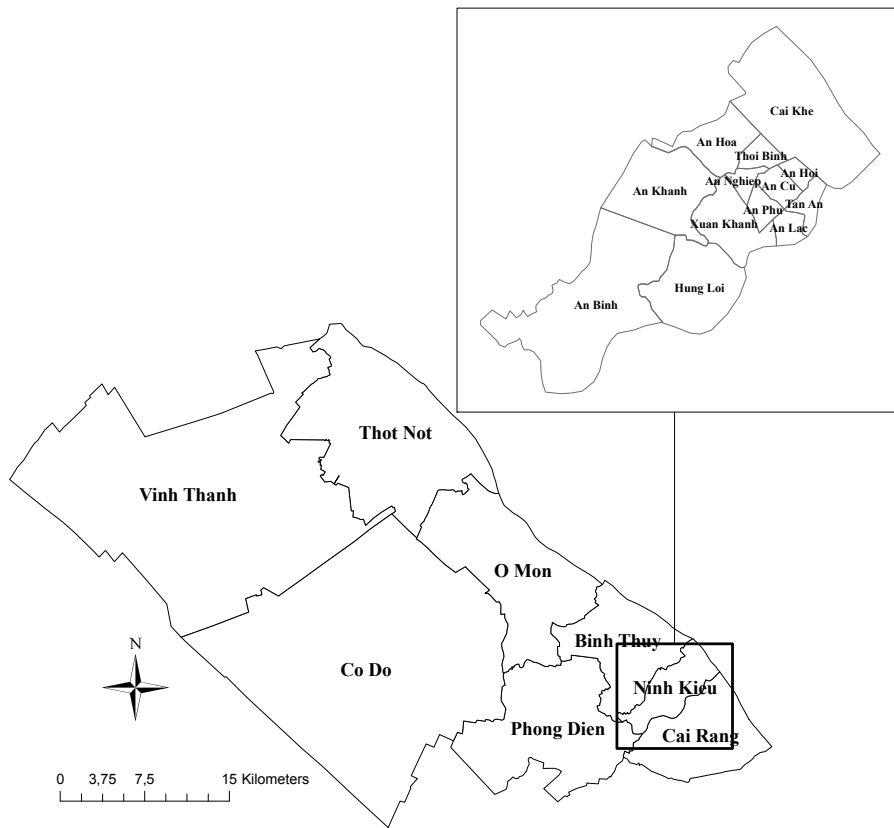


Figure 8. Map of Can Tho city province in the Mekong Delta region of Vietnam, showing the eight districts. The inset map shows the Ninh Kieu district (Can Tho city) with the 13 wards.

The selection of sampling periods was based on the yearly pattern of rainfall; mosquitoes were collected at the end of the dry period and at the end of the rainy period in 2009. The rationale behind this selection was to provide a representative descriptive picture of the mosquito population in a year, since a mosquito species benefiting from the dry season could be expected to be at its peak towards the end of this period, and vice versa.

Table 1. A description of the households included in paper II and III in the urban Ninh Kieu (NK) district or the rural Co Do (CD) district in Can Tho city province at the end of the dry season 2009 (sampling period 1) and at the end of the rainy season (sampling period 2).

Household	Ward	Pigs/1000 people	Sampling period	Pigs in household	People in household
A	An Binh (NK)	213	1	12	4
B	An Khanh (NK)	117	1	9	10
C	Hung Loi (NK)	25	1	10	8
D	Thoi Binh (NK)	0.8	1	11	7
E	Xuan Khanh (NK)	1.4	1	0	4
F	An Hoa (NK)	15	1	15	9
G	An Hoi (NK)	0	1	0	5
H	An Nghiep (NK)	0.5	1	0	4
I	Xuan Khanh (NK)	1.4	1	26	10
J	An Phu (NK)	1.9	1	35	7
K	An Nghiep (NK)	0.5	1	0	60
A	An Binh (NK)	213	2	34	4
C	Hung Loi (NK)	25	2	13	7
D	Thoi Binh (NK)	0.8	2	14	5
E	Xuan Khanh (NK)	1.4	2	0	4
F	An Hoa (NK)	15	2	25	8
I	Xuan Khanh (NK)	1.4	2	35	1
J	An Phu (NK)	1.9	2	16	5
K	An Nghiep (NK)	0.5	2	0	45
L*	Hung Loi (NK)	25	2	34	6
M	An Binh (NK)	213	2	15	4
N	An Binh (NK)	213	2	30	4
P	An Hoi (NK)	0	2	0	7
R*	An Binh (NK)	213	2	69	4
S	An Khanh (NK)	117	2	110	4
T	Cai Khe (NK)	8	2	4	6
U	Xuan Khanh (NK)	1.4	2	1	6
V*	Thoi La (CD)	41	2	81	2
X*	Thoi La (CD)	41	2	134	3
Z*	Thoi Hung (CD)	220	2	603	10

* Households only included in paper III

3.2 Questionnaires

In paper I the data had already been collected using questionnaires described by Boqvist *et al.* (2002).

In paper II, a questionnaire was used to collect data on household level. It is possible that the reported presence of poultry was biased by the fact that poultry keeping in the urban area is illegal. Therefore data on poultry was not used as a separate independent variable in the analyses. The questionnaire was repeated in both sampling periods, since changes in the household could occur. If pigs were sampled for paper III, a questionnaire to collect data on pig level was also added to the questionnaire with questions for the households.

3.3 Serological analyses (paper I and III)

In paper I, a commercial indirect IgG ELISA, (Shenzhen Lvshiyuan Biotechnology Co Ltd., Shenzhen, China) was used. In paper III both an IgM antigen capture (MAC) ELISA and a competitive IgG ELISA were used (Australian Animal Health Laboratories, Geelong, Victoria, Australia). The latter two ELISAs have been described previously (Williams *et al.*, 2001; Pant *et al.*, 2006).

A problem when comparing the results from different JEV seroprevalence studies is the variation in methods used for serological testing, and the extensive use of in-house tests (Koraka *et al.*, 2002). Therefore, the original aim of the study was to use an already validated, preferably commercially available ELISA, which could have been used in all studies and which would have been available to other research groups. The commercial ELISA used in paper I was, however, not available for the analyses for paper III.

It has been shown previously that heat-inactivation may lead to false positive reactions in ELISA for other viruses, including another virus in the Flaviviridae family, Hepatitis C virus, (Yoshida *et al.*, 1992). Thus controls consisting of Swedish samples that were assumed to be free from JEV were included. These Swedish pig sera were split into three parts; one part remained untreated whilst the other parts were treated at 56 °C for 30 min and 60 min respectively. In all ELISAs these were used as additional negative controls to ascertain that heat treatment did not affect the optical density (OD).

3.4 Entomological studies

Mosquitoes were collected during night time using un-baited CDC mini light traps (Figure 9), Bioquip Products, California, USA, with a 4 W light bulb as the sole attractant for the mosquitoes.

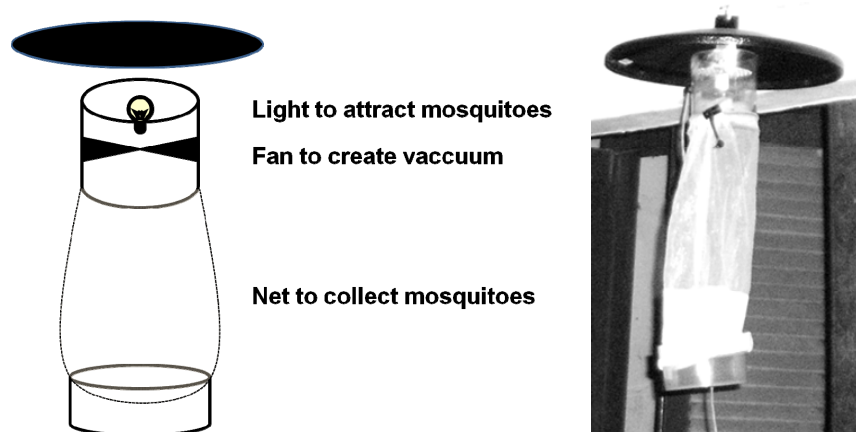


Figure 9. Schematic drawing of a CDC mini light trap and a picture of an affixed trap.

In a comparison with other types of traps, light traps have been shown to yield more species and more female mosquitoes (Chen *et al.*, 2011). Light traps are suitable for nocturnal mosquitoes, such as *Culex* species, which often feed from sunset to dawn (Gould *et al.*, 1974). Since *Culex tritaeniorhynchus* is an especially phototactic mosquito, light traps have been recommended for collection of this species (Reisen *et al.*, 1976), but then other species that are not equally attracted to light may be under-represented. The CDC mini light traps can also be baited with dry ice, which may increase the number of mosquitoes 2.5 times compared to un-baited traps (Leake *et al.*, 1986), but the CO₂ emitted may change the species composition of mosquitoes collected. The impact of CO₂, however, differs between studies; Chen *et al.* (2011) showed that more mosquitoes which had never eaten blood previously were collected in CO₂ baited traps than in the un-baited traps, and this was considered to lower the number of virus-positive mosquitoes, whereas Leake *et al.* (1986) found proportionally more mosquitoes carrying virus in baited traps. For practical reasons, the traps were not baited in papers II-III. Although this likely reduced the total number of mosquitoes collected than if traps had been baited, the species composition of *Culex* mosquitoes ought to be representative. It is, however, difficult to compare results from other areas where different methods were used.

Mosquitoes were identified morphologically, which may lead to misclassification. Several species in the *Cx. sitiens* group are known to be difficult to distinguish, and especially the difference between *Cx. vishnui* and *Cx. pseudovishnui* can be subtle and variable (Reuben *et al.*, 1994; Chapman *et al.*, 2000; Sim *et al.*, 2009). Therefore, only *Cx. tritaeniorhynchus* was identified to species level in this subgroup. As there are still risks of misclassifying this species (Hasegawa *et al.*, 2008), *Cx. tritaeniorhynchus* was

treated both as a species and as part of the *Cx. vishnui* subgroup in the statistical analyses. Genera apart from *Culex* were identified to genus level.

When more than 300 mosquitoes were collected in a trap, which occurred at two households, only 300 specimens were identified. To estimate the number of each species in the trap the following formula was used:

$$\text{Estimated number of Species X} = \frac{\text{Counted Species X} \times \text{Total number}}{300}$$

It was not always possible to make repeated mosquito collections at all households. In paper II, mosquito collections are included from all urban households where the light traps functioned at least on one occasion during the whole night, and thus two urban households were excluded, in order not to bias the statistical analyses. To compare households with and without pigs, traps were operated as close as possible to human dwellings. In households with pigs, a trap was also operated close to the pigs (Figure 10).

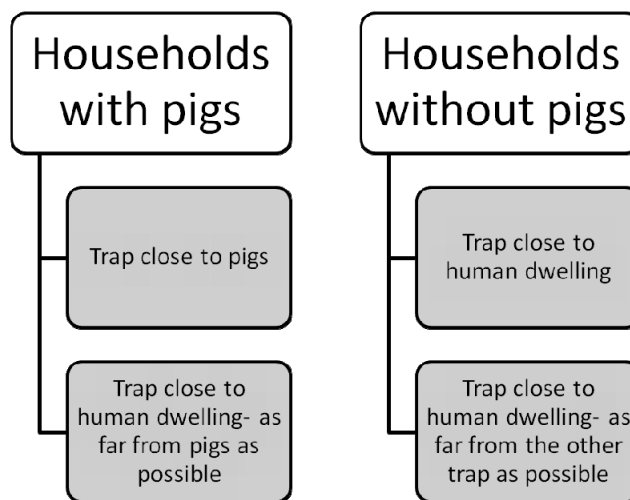


Figure 10. A schematic design of the strategy for placing mosquito traps to collect Japanese encephalitis virus vectors in Can Tho city, Vietnam.

3.5 Detection of Japanese encephalitis virus in mosquitoes

During the first trials to detect JEV in mosquito samples, a one step RT-quantitative, real-time, PCR (qPCR) was used with primers and probe according to Pyke *et al.* (2004). However, this PCR protocol suffered from problems with low sensitivity, and gel electrophoresis of the PCR products from mosquito samples only displayed smears. To increase the sensitivity a nested PCR was therefore established. Since it is known that inhibitory factors

present in mosquitoes may inhibit PCR reactions (Harris *et al.*, 1998; Lardeux *et al.*, 2008), an experiment with serial dilutions of spiked mosquito pools was conducted to evaluate the magnitude of the problems with inhibitions, and to verify that RNA dilution could work to circumvent the problem. Pools of male mosquitoes were included in the virological analyses in order to find possible evidence for vertical transmission.

A solution with phenol and guanidine isothiocyanate (Trizol, Invitrogen/Life sciences, California, USA) was added to the mosquito pools for four reasons. First, Trizol has been demonstrated to preserve RNA well, even after prolonged storage at room temperature (Hofmann *et al.*, 2000). Second, it has the capacity to inactivate viruses (Blow *et al.*, 2004). Third, it was possible to continue with Trizol extraction, which is a method that requires little equipment and is available in many low-income and tropical countries, and fourth, extractions with phenols yielded the least inhibition from mosquitoes when different extraction methods were compared by Townson *et al.* (1999).

3.5.1 Nested RT-PCR

Two previously published protocols, covering the same part of the NS5-3' untranslated region (UTR), were chosen for the nested RT-PCR in paper III (Figure 11). The 3'UTR region in JEV may vary between 560 and 585 base pairs (bp) (Weaver *et al.*, 1999) and there may be repeated sequences causing double bands to occur on gel electrophoresis after RT-PCR (Pierre *et al.*, 1994). The first RT-PCR was run with 50 cycles and the second inner qPCR with 40 PCR cycles. The Taqman probe used in the qPCR was modified from Pyke *et al.* (2004) with a central degeneration, since a previous isolate from the Can Tho city province (Thu *et al.*, 2006) displayed a variation at that site.

The sensitivity of the nested RT-PCR was compared to the one-step RT-qPCR used in the preliminary trials but using the modified probe. Qiagen One-Step RT-PCR (QIAGEN GmbH, Hilden, Germany) was used according to the manufacturer with 40 cycles, since this method gave the most sensitive detection during the preliminary tests.

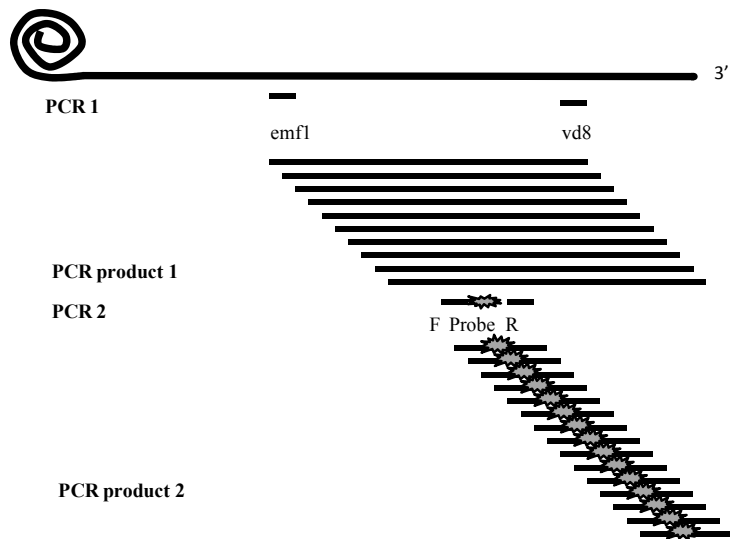


Figure 11. Schematic representation of the nested JEV RT-PCR in paper III. The primers emf1 and vd8 amplifies a long sequence of the NS5-3'-UTR region (Pierre *et al.*, 1994). The PCR product 1 was then used in a second reaction with the primers F and R (Pyke *et al.*, 2004).

3.5.2 Spiking of Swedish mosquitoes

Swedish mosquitoes were used in the experiment to evaluate PCR inhibition in paper III. These mosquitoes were not identified, but earlier studies have shown that *Aedes* and *Ochlerotatus* spp are the most numerous in Sweden (Schäfer & Lundström, 2001; Schäfer *et al.*, 2004), and it is assumed that the majority of mosquitoes used here were *Aedine*. Virus was diluted 1:100 and 1:1000 in Trizol solution and 1:1000 in the mosquito homogenates from the pools of five and 50 mosquitoes. These dilutions were extracted in the same way as the mosquito pools from Can Tho city. In addition, the RNA extraction of the 1:100 dilution in Trizol was diluted 1:10 in RNA extract from 50 non-spiked mosquitoes. In this way three different RNA extracts of spiked mosquitoes were achieved and these were diluted in a 10 series.

The viral concentration of the Nakayama strain used for spiking was unknown. Therefore sensitivity was not measured, but it was still possible to demonstrate the extent of the inhibition of the mosquitoes. Another way of spiking mosquitoes would have been to experimentally infect a mosquito and then add it to a pool of uninfected mosquitoes. However, this would have required high security laboratory facilities, and experimentally infected mosquitoes do not necessarily carry an amount of virus similar to naturally infected ones.

3.6 Sequencing and phylogenetic analyses

The PCR product from the first RT-PCR was amplified with the emf1 primer and the inner reverse primer (R), resulting in a 133 bp JEV fragment. Japanese encephalitis virus strains from the NCBI GenBank database, representing all five genotypes, were used as references in the phylogenetic analysis. Since genotypes are based on the prM and E genes (Uchil & Satchidanandam, 2001) alignment and phylogenetic analysis were also performed with the references strains at full length.

3.7 Data analyses and spatial mapping

In paper I and II different subsets of models were built in the statistical analyses (Table 2). In all models, manual backward elimination was used instead of automatic to control for confounders. The reason for including both the serological results as positive/negative and the OD-values obtained from the ELISA used in paper I, was that high OD-values can reflect high antibody titres, which may be associated with a more recent infection (Burke *et al.*, 1985b), or may be associated with repeated infections.

Additional analyses not reported in the papers, included a comparison between the resulting seroprevalence in paper III and the seroprevalence in paper I, using Fisher's exact test. Multivariable analyses on the reproductive performance on the pigs in paper III, were also performed, but did not include serological results.

The minimum infection rate (MIR) in the mosquitoes was calculated as the number of positive pools divided by the total number of mosquitoes and assumes that at minimum, there is one positive mosquito per positive pool. For comparison, calculations of the infection rate were also done using maximum likelihood estimate (MLE), which is considered to be more robust and exact than MIR (Gu *et al.*, 2003). Maximum likelihood estimate calculations are not as straightforward as the calculation of MIR; and thus the software by Biggerstaff (2005) was used. Since MIR provides a minimum number it is expected to always be less than MLE.

Household coordinates were registered with Garmin Geko™ 201 and spatial mapping was performed using ESRI® ArcMap™ 9.3.1.

Table 2. The different model subsets and variation in variables used in the statistical analyses in paper II and III, used to analyze the risk factors associated with increased JEV-seropositivity (paper I), the association between JEV serological results and reproductive performance (paper I), and mosquito abundance and household and ward factors (paper II).

Dependent variable	Subset models	Variation in independent variables	SAS procedure
JEV-seropositivity	All sows		Glimmix
Alive born piglets	Sows < 1.5 years	Seropositive/negative or OD-values	Mixed
	All sows	Seropositive/negative or OD-values	Mixed
Stillborn piglets	Sows < 1.5 years	Seropositive/negative or OD-values	Glimmix
	All sows	Seropositive/negative or OD-values	Glimmix
<i>Culex tritaeniorhynchus</i>	Only households with pigs		Mixed
	All households, only collections close to humans	Number of pigs or presence of pigs	Mixed
<i>Culex vishnui</i> subgroup	Only households with pigs		Mixed
	All households, only collections close to humans	Number of pigs or presence of pigs	Mixed
<i>Culex gelidus</i>	Only households with pigs		Mixed
	All households, only collections close to humans	Number of pigs or presence of pigs	Mixed
<i>Culex quinquefasciatus</i>	Only households with pigs		Mixed
	All households, only collections close to humans	Number of pigs or presence of pigs	Mixed
<i>Culex</i> males	Only households with pigs		Mixed
	All households, only collections close to humans	Number of pigs or presence of pigs	Mixed
Blood-filled mosquitoes	Only households with pigs		Mixed
	All households, only collections close to humans	Number of pigs or presence of pigs	Glimmix
			Glimmix

4 Main results and discussion

4.1 Animal farming in Can Tho city province

The eight districts of Can Tho city province have an increasing human population closer to the urban Ninh Kieu district, which constitutes Can Tho city (Figure 12). Rice production is more intense in the rural districts (Figure 13). The two districts with the most rice growing, Co Do and Vinh Thanh district, had the lowest pig densities (Figure 14) in 2008 (Anh, 2009).

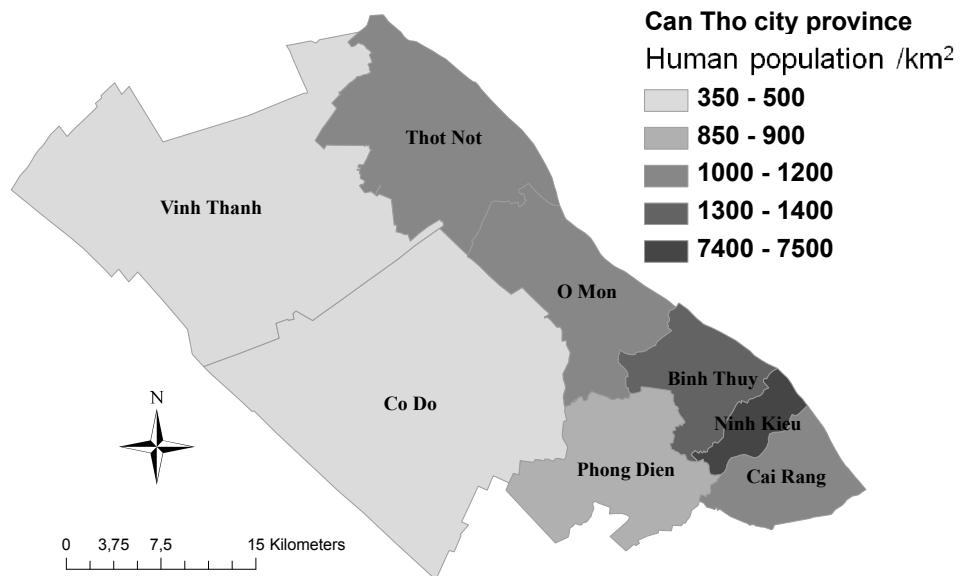


Figure 12. Human population density in the eight districts of Can Tho city province in 2008.

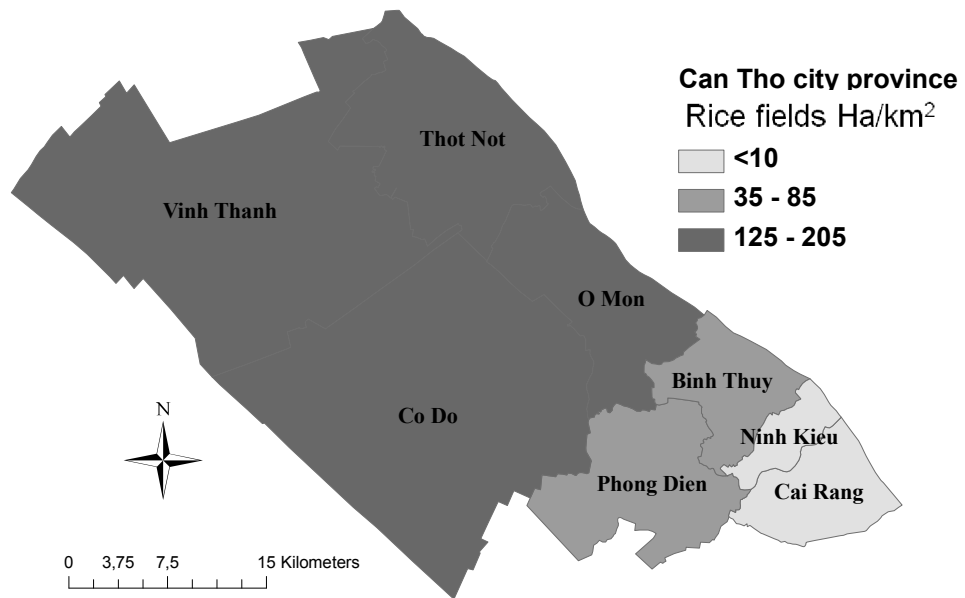


Figure 13. Rice field hectares grown during 2008 (max three harvests per year) per km² in the eight districts of Can Tho city province.

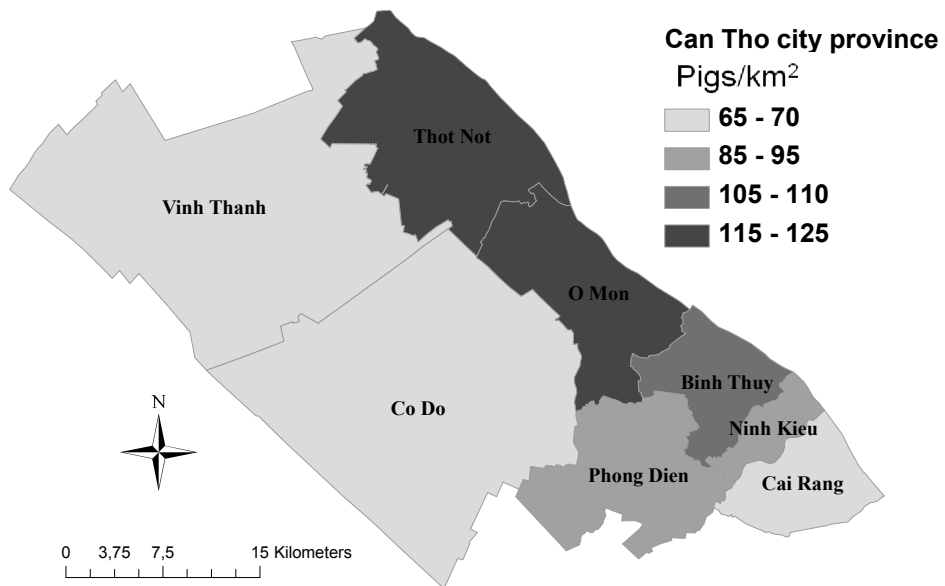


Figure 14. Pig density in the eight districts of Can Tho city province in 2008.

Within the Ninh Kieu district 288 households kept pigs in 2008, with a total of 4 794 pigs being reported. Pigs were the most common livestock kept within

the city. In the wards that kept pigs, the pig densities varied between 14 and 340 pigs/ km² in 2008, and between 17 and 302 pigs/ km² in 2009. Ninh Kieu district has a human population of more than 200 000 inhabitants, and in the whole district there were 20 pigs per 1000 urban inhabitants in 2008. The ward with most pigs had 213 pigs per 1000 urban inhabitants. The pig density is plotted against the human population density in figure 15.

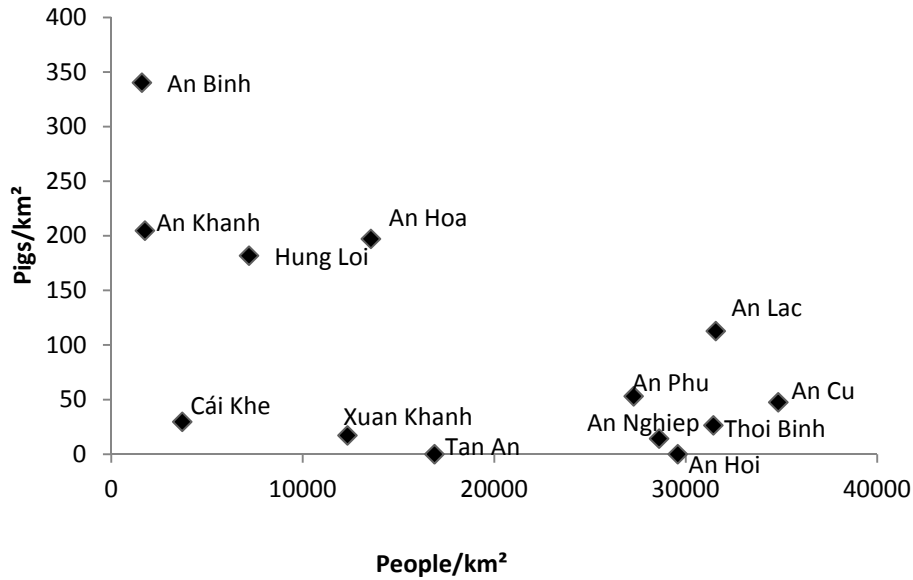


Figure 15. Pig density plotted versus human population density in the 13 wards in Ninh Kieu district (Can Tho city).

4.2 Seroprevalence in female pigs (Papers I and III)

Of the 315 sows sampled in 1999, 190 (60%) were seropositive (paper I). There were significant differences in both seroprevalence and the mean sample OD-values between the four farms in the study, as well as between the breeds and age categories (Table 3). There was however no significant difference depending on the month of sampling.

Table 3. Seroprevalence of JEV and mean sample optical density (OD) value in 315 sows sampled in 1999, from four different farms, of different breed, different age and sampled during different periods.

Farm	A	B	C	D	P
Positive %	65	67	31	79	<0.0001
Mean OD-value	0.95	0.93	0.44	1.00	<0.0001
Breed	Duroc	Landrace	Yorkshire	Hybrids	P
Positive %	40	57	68	51	0.0467
Mean OD-value	0.54	0.75	0.96	0.66	0.0017
Age category	<1.5 year	1.5-3.5	>3.5 years	P	
Positive %	53	62	78	0.0106	
Mean OD-value	0.66	0.80	1.29	<0.0001	
Month	March	August	December	P	
Positive %	54	63	64	0.2235	
Mean OD-value	0.76	0.87	0.85	0.4054	

Out of the 74 pigs sampled in 2009 in Ninh Kieu and Co Do district, 73 (99%) were JEV seropositive and one pig tested inconclusive (paper III). This is in accordance with other seroprevalence studies in endemic areas. Studies in Cambodia showed JEV seropositivity of 95% in pigs above six month old (Duong *et al.*, 2011). Newly introduced pigs in an endemic area can be infected within days and subsequently seroconvert within weeks (Burke *et al.*, 1985c). All 43 pigs that lived within the Ninh Kieu district were seropositive and, according to the owners, at least 24 had been born in the same urban household where they were sampled. These pigs can thus be assumed to have been infected within the city. It is possible that more of the pigs had been born within the city as well, although this could not be verified. Infection with JEV has previously been demonstrated in humans in cities (Bi *et al.*, 2007; Vallée *et al.*, 2009), but in contrast to humans who move in and out of cities, pigs in a household are assumed to be stationary, and seroconversion in pigs may thus be a better indication of actual transmission of JEV within the city.

No pigs sampled in 2009 were positive in the IgM ELISA. Considering that IgM levels in pigs are at a maximum one week after infection, after which they decrease (Burke *et al.*, 1985a), the likelihood of detecting IgM positive pigs is low in endemic areas, which may explain this result.

The seroprevalences in paper I and III were significantly ($p < 0.0001$) different. Several explanations can be put forward for this difference. First, there were ten years between the samplings. In a region where the pig and rice

production is growing it may be expected that JEV exposure will increase, (Vo-Tong *et al.*, 1995; Erlanger *et al.*, 2009), which may result in higher seroprevalence.

Second, all sows in paper I were kept on pig farms in rural areas around Can Tho city, whereas 43 of the pigs in paper III were within the actual city. It is possible that there are differences in vector compositions and abundance between rural and urban pig holdings that were not discovered here, and that the transmission of JEV is in fact more intense within the city.

Third, two different ELISAs were used, and it is possible that both sensitivity and specificity differed between them. Unfortunately it was not possible to compare the two ELISA methods. The number of pigs sampled in 2009 was also relatively low, although they originated from more farms than in paper I, and the average age of the pigs were lower (mean age 23 months in 2009 versus 30 months in 1999).

Apart from JEV, the only known members of the family Flaviviridae that infect mammals in Vietnam are Dengue Virus and Hepatitis C Virus (Bartley *et al.*, 2002). Dengue virus is also in the flavivirus genus, similarly to JEV, but in another serological group (Calisher *et al.*, 1989), and is not primarily a swine pathogen. The serological response to natural infection of Dengue virus in pigs has, therefore, not been elucidated, but in experimental infection with Dengue virus in pigs it has been possible to detect antibodies (Burgess *et al.*, 2006; Tjaden *et al.*, 2006). However, JEV IgG antibodies cross-react less with Dengue than against viruses in the JEV serological group (Kimura-Kuroda & Yasui, 1986).

4.3 Reproductive performance (Papers I and III)

The average total number of piglets born per litter by the 315 sows sampled in 1999 was 10.1, and the average number of piglets born alive was 9.2. In the sows sampled in 2009 the average total number of piglets born was 9.9 and the average born alive was 9.1.

In paper I the number of piglets born alive was analyzed instead of total born piglets, since it is more relevant for pig producers. The OD-values only showed a significant association with the number of piglets born alive in interaction with breed. In epidemic areas where JEV is suspected to cause economic disadvantages to the pig producers, it might be worth further studying if there are any biologically significant differences between pig breeds, especially if hybrids are less affected than pure breeds, as indicated in the present study.

When all sows were analyzed, the OD-values did not show any association with the number of stillborn piglets, but when the analyses were limited to sows less than 1.5 years, a one unit increase in OD-value was associated with 0.88 more stillborn piglets. In spite of the known teratogenic effects of JEV (Burns, 1950; Shimizu *et al.*, 1954) there is an apparent lack of impact on the reproductive capacity in the sows in this study. One explanation for this could be that reproductive symptoms are mainly observed when non-immune pregnant pigs are infected before maternal immunocompetence (Gresham, 2003). The maternal protection of antibodies usually wanes before half a year of age (Hale *et al.*, 1957; Scherer *et al.*, 1959c) and first mating commonly occurs at seven to 10 months of age in Vietnam (at second oestrus) (Dan & Summers, 1996). In areas where JEV occurs seasonally (Watanabe, 1968; Kawakubo *et al.*, 1969; Takashima *et al.*, 1988), all pigs born after the epidemic would be immunologically unprotected at the time of the next outbreak and their first or second pregnancy. A study in Japan showed that if gilts had time to be exposed to the virus and produce antibodies before mating, their reproductive results were as good as vaccinated gilts and much better than gilts that were infected during pregnancy (Kawakubo *et al.*, 1969). Intensive transmission of JEV in an endemic area, such as Can Tho city, is expected to ensure that more gilts are infected before onset of puberty and fewer remain susceptible after first mating.

There was a significant positive association between the mean OD-value on the farm, reflecting the immune status of the farm, and the number of piglets born alive. This supports the theory of a high infection pressure, causing immunity early in life with continuous boosting, and consequently fewer clinical signs. A similar association between low morbidity in humans and high infection pressure has been observed for Dengue virus (Nagao & Koelle, 2008).

The average number of piglets born in total and alive by the 51 sows sampled in 2009 is shown in table 4. In multivariable analyses a significantly ($p=0.008$) lower number of matings was required for pigs in rural households than in urban households, which likely is explained by the fact that the rural households had more pigs and at a more professional level. Increasing parity, up to parity three, was significantly associated with both increased number of matings ($p=0.004$) and number of piglets born alive ($p=0.018$). No statistical analysis was performed to test the association between JEV seropositivity and reproduction, since the seropositivity was close to 100%.

Table 4. The average number of piglets born in total and born alive, in the sows sampled in 2009 and included in paper III.

	Total number of piglets born	Piglets born alive
Hybrids	10.6	9.6
Pure-bred	9.6	8.9
Co Do	10.8	9.4
Ninh Kieu	9.1	8.9

According to the pig keepers, almost half (23/51) of the parous sows had shown at least one symptom that could be caused by JEV or another pathogen causing reproductive failures. Stillborn or mummified foetuses were reported in 29%, and weak-born piglets in 24%. Almost one third of the sows had shown repeated oestrus and 8% of the sows had aborted at least once, but it is not possible to conclude that the symptoms were related to JEV infection. A number of other reproductive pathogens are present in the Mekong delta region, such as classical swine fever, porcine reproductive and respiratory syndrome, pseudorabies, leptospirosis and parvovirus (Boqvist *et al.*, 2002; Kamakawa *et al.*, 2006). Piglets with neurological symptoms, such as shivering, were reported for 4% of the sows, but such symptoms are not pathognomonic for JEV. In the questionnaires, households had been asked if they vaccinated their pigs, and against which diseases, but the majority of the households could not answer this question, and the presence of a local veterinarian at the time of the interview could cause a bias. Therefore these variables were not analyzed.

4.4 Mosquito collections (Papers II and III)

In paper II, 7419 mosquitoes from 17 urban households were analyzed, and in paper III, 7885 mosquitoes from 19 urban and three rural households were analyzed. The mosquitoes collected per household are displayed in table 5.

Culex tritaeniorhynchus was the most abundant mosquito collected in Can Tho city, followed by *Cx. gelidus* and *Cx. quinquefasciatus*. Only a few mosquitoes were classified as being other members of the *Cx. vishnui* subgroup, and the same results were achieved when *Cx. tritaeniorhynchus* was analyzed by itself as when the entire *Cx. vishnui* subgroup was analyzed. Interfering light may affect the collections (Moore & Gage, 2005) and is more likely a problem in cities than in rural areas. This may have added to the variation between collections (Table 6). To draw conclusions on seasonality, data from several years would have been required, and since the present study was limited to 2009, seasonality was not analyzed.

Table 5. All mosquitoes collected during the spring and fall 2009 in Can Tho. All mosquitoes were included in paper III, whereas household L,R,V,X and Z were excluded from paper II.

Species/Household	A	B	C	D	E	F	G	H	I	J	K	L	M	N	P	R	S	T	U	V	X	Z	Total
<i>Culex tritaeniorhynchus</i>	226	3	179	110	4	75	3	2	339	72	3	1	46	6	1	1	545	124	1	35	103	2	1881
<i>Culex vishnui</i> subgroup*	41	1	26	12	3	12	1	-	11	6	-	-	4	-	-	-	5	-	-	4	11	-	137
<i>Culex gelidus</i>	274	18	237	93	2	64	2	1	264	37	1	3	55	3	8	8	77	24	-	64	21	1	1249
<i>Culex quinquefasciatus</i>	18	6	67	184	68	104	9	-	6	60	11	1	2	-	4	1	1	200	10	-	36	-	788
<i>Culex fuscocephala</i>	2	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5
<i>Lutzia</i> sp	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
<i>Anopheles</i> spp	54	9	53	16	2	7	1	-	33	4	1	-	3	6	11	76	28	1	2	10	-	-	317
<i>Mansonia</i> spp	15	3	56	3	-	3	1	-	48	-	-	-	2	3	-	17	1	-	2	7	-	-	161
<i>Aedes</i> spp	1	-	8	1	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-	12
<i>Uranotenia</i> spp	14	3	4	1	-	1	-	-	-	-	-	-	8	-	-	10	-	-	-	-	-	-	41
<i>Culex</i> males	26	4	71	81	55	111	10	4	13	54	10	2	-	3	1	11	82	12	2	125	1	-	678
<i>Anopheles</i> males	-	1	-	1	-	-	-	-	1	-	-	-	-	-	-	-	2	2	1	-	1	-	9
<i>Mansonia</i> males	5	3	7	-	-	-	-	-	26	-	-	-	-	-	-	-	3	-	-	-	1	2	47
<i>Aedes</i> males	1	-	-	-	-	1	-	-	-	-	1	-	-	-	-	-	-	-	2	-	-	-	5
Unidentified	20	-	6	20	2	1	-	-	874	1	-	-	2	-	-	2	1605	15	1	1	4	-	2554
Total	697	52	717	522	136	379	27	8	1615	234	27	7	122	21	5	24	2353	476	28	110	319	6	7885

* The *Culex vishnui* subgroup does not include *Culex tritaeniorhynchus* in this table

Table 6. Variation in the number of mosquitoes collected in Can Tho city, Vietnam, in 2009.

Total number of mosquitoes	Median	Mean	Range
Households without pigs	4	10	1-81
Household with pigs, traps close to humans	12	39	1-452
Household with pigs, traps close to pigs	58	160	1-1901
Total number of <i>Culex tritaeniorhynchus</i>	Median	Mean	Range
Households without pigs	0	0.6	0-2
Household with pigs, traps close to humans	3	15	0-314
Household with pigs, traps close to pigs	11	79	0-1478
Total number of <i>Culex gelidus</i>	Median	Mean	Range
Households without pigs	0	0.3	0-2
Household with pigs, traps close to humans	2	5	0-53
Household with pigs, traps close to pigs	13	39	0-342
Total number of <i>Culex quinquefasciatus</i>	Median	Mean	Range
Households without pigs	2	5	0-43
Household with pigs, traps close to humans	2	8	0-72
Household with pigs, traps close to pigs	3	11	0-132
Total number of <i>Culex</i> males	Median	Mean	Range
Households without pigs	1	4	0-37
Household with pigs, traps close to humans	3	6	0-28
Household with pigs, traps close to pigs	3	9	0-56
Proportion blood-filled females	Median	Mean	Range
Households without pigs	0	0	0-0.38
Household with pigs, traps close to humans	0.17	0.17	0-0.67
Household with pigs, traps close to pigs	0.37	0.35	0-0.67

4.4.1 Risk factors associated with presence of vectors

The association between factors at household and ward level and the numbers of urban mosquitoes was analyzed in paper II. No risk factors were associated with the numbers of *Culex* males or the proportion of blood filled mosquitoes.

Pigs and other livestock

The number of pigs kept in households was positively associated with the total number of mosquitoes collected, and the number of *Cx. tritaeniorhynchus*. Traps operated close to pigs contained significantly more mosquitoes in total, and more *Cx. tritaeniorhynchus* and *Cx. gelidus*, compared to traps operated close to humans. The role of pigs as amplifiers for JEV makes them an

important risk factor for JEV infection. Pig density has previously been observed to be associated with the number of human JE cases (Hsu *et al.*, 2008) and with higher seroprevalence in pigs (Yamanaka *et al.*, 2010). The presence of pigs in households has also been shown to be associated with increased risk for JE in humans (Liu *et al.*, 2010).

In this study it seemed as if the presence of other livestock had little influence on the number of *Cx. tritaeniorhynchus* found in a household. This zoophilic species is known to feed extensively on both large ruminants and pigs (Reuben *et al.*, 1992; Bhattacharyya *et al.*, 1994; Arunachalam *et al.*, 2004). Although feeding preferences were not analyzed here, the strong association between this variable and the number of pigs indicates that pigs are important for the presence of *Cx. tritaeniorhynchus* in an urban area. However, in the present study *Cx. tritaeniorhynchus* could be identified in every ward where mosquitoes were collected, and were not dependent on the presence of pigs.

Culex tritaeniorhynchus is not only one of the most important vectors for JEV, but may harbour other arboviruses as well (Leake *et al.*, 1986; Ha *et al.*, 1995; Wang *et al.*, 2011). Many of these viruses can infect humans or animals and some are newly discovered, with unknown disease potential. The ubiquitous presence of *Cx. tritaeniorhynchus* within Can Tho city thus not only implies a risk for urban JEV transmission but also the potential for transmission of other pathogens.

In contrast to the *Cx. vishnui* subgroup, *Cx. gelidus* was not associated with the number of pigs in the household, but rather with the pig density in the ward. In addition, *Cx. gelidus* was the only species that was positively associated with the presence of other livestock in the household. The strong preference of this species for feeding on cattle (Reuben *et al.*, 1992) could be the explanation for this observation and *Cx. gelidus* has previously been shown to be associated with presence of cattle (Hasegawa *et al.*, 2008).

Increasing the number of cattle, which are not capable of amplifying JEV, in order to divert the vectors from pigs and humans, is referred to as zooprophyllaxis. An increased cattle to human ratio has, for example, been shown to decrease the number of *Cx. tritaeniorhynchus* feeding on humans (Gajanana *et al.*, 1995) and the preference of *Cx. gelidus* for cattle could cause the same effect. Zooprophyllaxis using cattle, however, is reported to work optimally only if there are limited larval habitats (Farajollahi *et al.*, 2011). Here it seems that the number of *Cx. gelidus* is positively associated with the presence of livestock, both close to pigs and close to humans. This indicates that access to breeding grounds is no limitation within the city, and that keeping cattle in the city may actually exacerbate the vector problems.

Presence of rice fields and fish ponds

The presence of rice fields and fish ponds only influenced the number of *Cx. gelidus*. *Culex gelidus* is known to breed in a wide variety of sites (Whelan *et al.*, 2000) and larval habitats have been previously identified close to human habitats (Hasegawa *et al.*, 2008). There was no association between *Cx. tritaeniorhynchus* and the presence of near-by rice fields in our study. *Culex tritaeniorhynchus* is known to exploit rice fields as larval habitats (Takagi *et al.*, 1997; Tsai, 1997), but it seems here that abundance of this species is independent of rice fields in the city. There could be several explanations for this observation. This mosquito species can disperse widely by flying or by wind (Wada *et al.*, 1969; Kay & Farrow, 2000), and could, therefore, possibly be found far from its breeding ground. *Culex tritaeniorhynchus* has, however, also been reported to use different larval habitats, as long as there is fresh water and vegetation (Colless, 1955). It is therefore more likely that *Cx. tritaeniorhynchus* exploits alternative breeding grounds within the city, such as ground pools of stagnant water, which are present all year in Can Tho city.

Humans

The number of people in the household was positively associated with the total number of mosquitoes and the number of *Cx. quinquefasciatus* in traps close to humans. This is consistent with the previous findings that this species is anthropophilic (Reuben *et al.*, 1992; Zinser *et al.*, 2004) and a common mosquito in households in Ho Chi Minh City (Huber *et al.*, 2003).

The JEV vector competence of *Cx. quinquefasciatus* is regarded as low compared to *Cx. tritaeniorhynchus*, (Reeves *et al.*, 1946; Sirivanakarn, 1976; Reuben *et al.*, 1994; van den Hurk *et al.*, 2003), but it was one of the species in South Vietnam from which most JEV isolates were made at the end of the 20th century (Do *et al.*, 1994). Since vector competence is not as important for vector capacity as the vector abundance and human biting rate (Dye, 1986; Kramer & Ebel, 2003), a poor vector, such as *Cx. quinquefasciatus*, can be of great epidemiologic importance. Even though *Cx. quinquefasciatus* is anthropophilic, it has been observed that this vector can have mixed blood meals from humans and pigs (Hasegawa *et al.*, 2008), proving that the same mosquito individual may bite both hosts, and thereby act as a bridge vector for transmission to humans. The species can also transfer JEV vertically (Hurlbut, 1950; Rosen, 1989).

4.5 Establishment of a nested RT-PCR protocol (Paper III)

The nested RT-PCR was able to detect JEV in all dilutions tested with pure extracted JEV, whereas the one-step RT-qPCR could not detect viral RNA in extracts diluted more than 1:100, nor detect viral RNA in any of the spiked mosquito samples (Table 7). The nested RT-PCR could only detect viral RNA in spiked mosquito samples if the RNA was diluted. The same results were achieved if extracted JEV-RNA was added to the RNA extracts from the mosquito pools, as if virus was extracted with the mosquitoes. This indicates that the inhibition occurs in the RT-PCR reaction and not in the preceding extraction step.

Table 7. Evaluation of the inhibitory effect of mosquitoes on a JEV RT-PCR and comparison of the sensitivity of one-step RT-PCR and nested RT-PCR.

RNA extract	Dilution	Nested Positive/runs	RT-PCR Mean ct*	One-step Positive/runs	RT-PCR Mean ct*
JEV diluted 1:1000 in Trizol	1:1	2/2	14.7	3/3	35.7
	1:10	2/2	16.6	3/3	40.3
	1:100	3/3	17.2	1/3	42.9
	1:1 000	3/3	21.2	0/3	
	1:10 000	2/2	26.3	0/3	
	1:100 000	2/2	28.6	0/3	
Extracted JEV, diluted 1:10 in the extract of 50 mosquitoes	1:1	0/2		0/3	
	1:10	0/2		0/3	
	1:100	2/2	25.9	0/3	
	1:1 000	2/2	22.2	0/3	
	1:10 000	2/2	29.7	0/3	
	1:100 000	2/3	34.7	0/3	
JEV diluted 1:1000 in homogenate of 5 mosquitoes	1:1	0/3		0/3	
	1:10	2/3	34.0	0/3	
	1:100	3/3	22.0	0/3	
	1:1 000	2/3	23.2	0/3	
	1:10 000	3/3	31.2	0/3	
	1:100 000	1/3	34.2	0/3	
JEV diluted 1:1000 in homogenate of 50 mosquitoes	1:1	0/2		0/3	
	1:10	0/2		0/3	
	1:100	2/2	20.6	0/3	
	1:1 000	2/2	24.6	0/3	
	1:10 000	2/2	29.6	0/3	
	1:100 000	2/2	36.4	0/3	

* Mean cycle threshold for the positive runs

Since all mosquitoes used for spiking were uninfected, the inhibition of the PCR could not be due to RNAi. Many factors have been postulated to be possible inhibitors, such as haem, polysaccharides or the pigment in the mosquito's eyes (Townson *et al.*, 1999; Nimmo *et al.*, 2006). The Swedish mosquitoes used in this spiking experiment were not visibly blood-filled, making it less likely that the inhibition was caused by ingested blood.

The level of inhibition was correlated to the concentration of mosquitoes. Similarly, in a PCR system for Rift Valley fever it was possible to detect one infected mosquito in a pool of 10, but not in pools of 25 or 50. However, repeated RNA extraction enabled detection in all pools (Ibrahim *et al.*, 1997).

Inhibitors present after extraction are a known major barrier in the detection of pathogens in its vector (Lardeux *et al.*, 2008). The results in the present study confirm that mosquito surveillance with PCR detection, without prior virus cultivation, must account for the risk of inhibition from the mosquitoes. Arboviruses are common in tropical, low-income countries and methods for detection, diagnostics and screening must be affordable, sensitive and robust (Mabey *et al.*, 2004). In this study, Trizol was used as diluent for its inactivating and preserving capacities. AgPath-id One-step RT-PCR and Path-id PCR kits were used in the nested RT-PCR for their simple protocols and comparatively low price, and have also been shown to have high sensitivity (Osman *et al.*, 2012). Different methods to circumvent the inhibition of mosquitoes in PCR have previously been described, including special extraction methods using magnetic or silica beads (Lanciotti *et al.*, 1992; Harris *et al.*, 1998), but these methods may be too expensive to be used extensively in many laboratories, or may yield too little RNA. As the pigment in the mosquito's eyes has been suggested to cause PCR inhibition, individual decapitation has also been used to improve detection (Nimmo *et al.*, 2006), but this may be too laborious in large screening programs. The outcome of the present study demonstrates that sample dilutions may be a simple and cost effective way to avoid PCR inhibition caused by mosquitoes. Also, if the mosquitoes are already homogenized or the RNA is already extracted, sample dilution may be a suitable way to proceed when mosquito inhibition is suspected to be a cause of PCR failures.

4.6 Detection of Japanese encephalitis virus in mosquitoes

Seven mosquito pools, out of 352 pools tested in paper III, were positive in the nested RT-PCR. All positive pools originated from traps close to pigs. Six of these pools were collected in Can Tho city, whereas one was from a pool in the rural Co Do district. Three pools contained *Cx. tritaeniorhynchus*, one

contained *Cx. quinquefasciatus*, and three pools contained unsorted mosquitoes.

Minimum infection rate (MIR) was 0.98 per thousand female mosquitoes and maximum likelihood estimate (MLE) was 0.99. In *Cx. tritaeniorhynchus* the MIR was 1.59 per thousand female mosquitoes and in *Cx. quinquefasciatus* MIR was 1.27 per thousand female mosquitoes.

Similar infection rates have been observed in other studies. In Taiwan mosquitoes were collected in a high risk area for JEV and an MLE of 0.87 per 1000 *Cx. tritaeniorhynchus* was estimated (Yang *et al.*, 2010). Some studies have shown much lower infection rates. In a study in suburban Bangkok, MIR was 0.05 per 1000 *Cx. tritaeniorhynchus* and 0.07 per 1000 *Cx. gelidus* (Gingrich *et al.*, 1987), and in the Can Tho province MIR was 0.05 per 1000 mosquitoes (Thu *et al.*, 2006). Both these studies used virus isolation as a detection method, which may have a lower sensitivity than PCR. There are also studies reporting much higher infection rates. In Korea the MLE of JEV infection rate was estimated to be 9.7 per 1000 mosquitoes (Kim *et al.*, 2011).

4.6.1 Phylogenetic analysis

The sequences from the positive samples were 90-99% identical (Table 8). The sequences clustered with isolates classified as genotype I and III. Genotype III was previously the most dominant in most Asian countries, but has gradually been replaced by genotype I, which is estimated to be the youngest genotype (Pan *et al.*, 2011). In Thailand the genotype shift and decrease in genotype III incidence has been temporally associated with increased industrialization of pig production and concurrent vaccinations with vaccines based on genotype III (Nitattapattana *et al.*, 2008). All the JEV reference strains clustered within the genotype they were reported to belong to, and the same way when analyzed at full length as when in the alignment with the 133 bp JEV fragments obtained in this study (Figure 16).

In Northern Vietnam, genotype I has gradually been replacing genotype III as the dominant genotype (Nga *et al.*, 2004). The present study provides the first indication of co-circulation in the Mekong Delta, and also in a geographically small area, the city of Can Tho. These findings also contradict the statement by Nabeshima *et al.* (2009) that genotype III has disappeared from Vietnam.

Sequences similar to genotype I were found in mosquito pools from one rural household and from two urban households, approximately 20 kilometres away. One of these urban households regularly purchases fattening pigs. Purchase of pigs could be an explanation for the spread of a viral strain of JEV from a rural area into the city, where it could easily spread further by urban vectors. It is also possible that an infected mosquito could transfer into the city either by flying or in a vehicle.

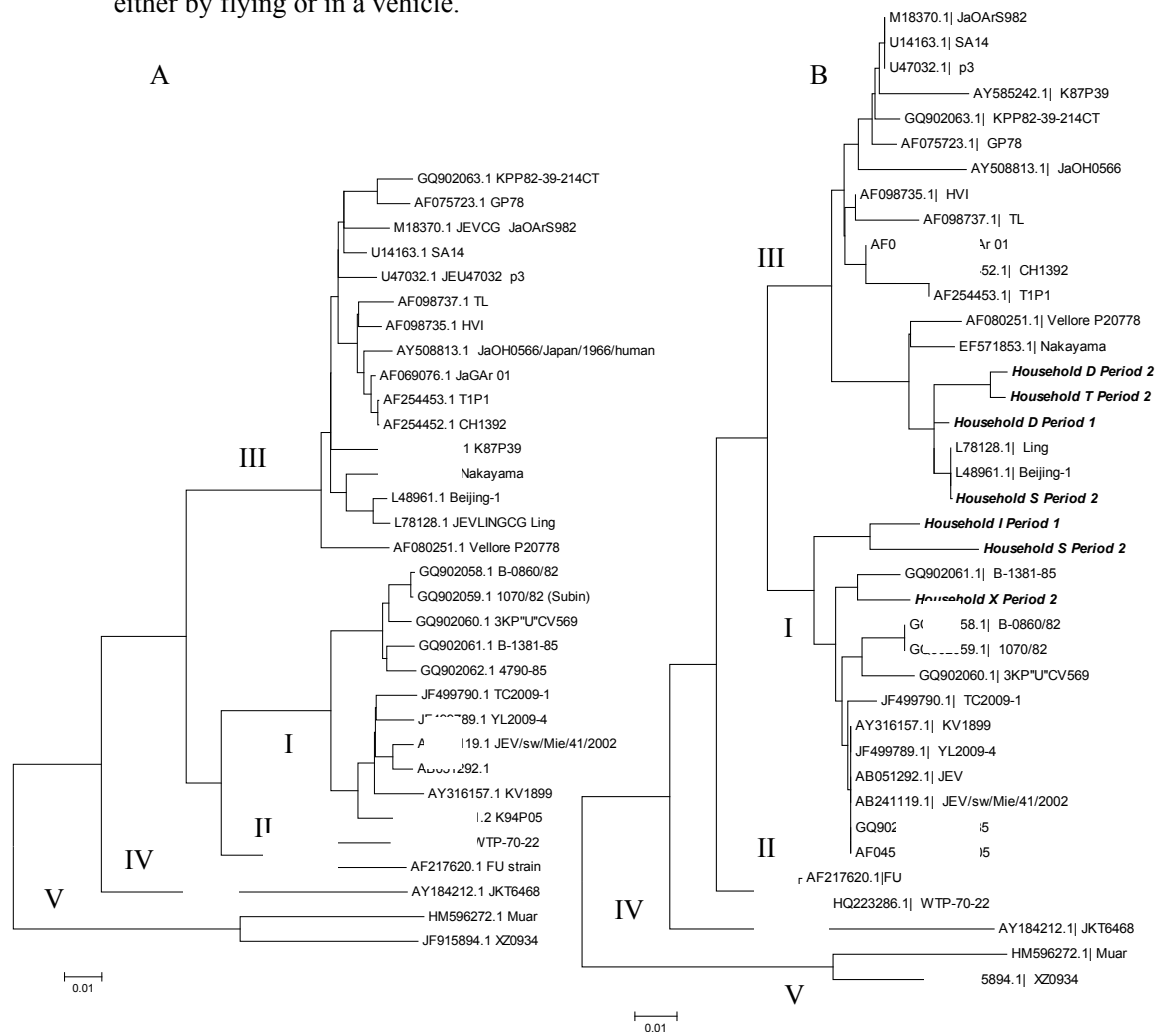


Figure 16. Phylogenetic trees of the five JEV genotypes. A: Phylogenetic tree based on the entire sequences of JEV reference strains. B: Phylogenetic tree with the seven JEV fragments from mosquito pools collected in Can Tho city. The end of dry season 2009 = sampling period 1, the end of the rainy season 2009 = sampling period 2.

5 Conclusions

This thesis provides new knowledge of JEV transmission in a tropical region where JE is endemic. The area studied in detail here is the Mekong delta in Southern Vietnam, with focus on the urban area of Can Tho city.

- The JEV seroprevalence in pigs in the Mekong delta is high, from 60 to 99%. Within Can Tho city 100% of the pigs had antibodies against JEV, including pigs that originated from the city. This indicates that there is a transmission of JEV from mosquitoes to vertebrate hosts within Can Tho city.
- Early exposure of most gilts to JEV, with an early sero-conversion, could be an explanation to why it was not possible to find an association between seropositivity and the number of piglets born alive, and to why an association between stillborn piglets and seropositivity could only be found in sows less than 1.5 years of age. Many of the gilts are therefore expected to be immune at the time of first pregnancy in this endemic area with a high infection pressure.
- Can Tho city is densely populated, and pigs are the most common livestock kept in the urban agriculture. Within the city, the more peripheral wards with the lowest people density had the highest pig density. Rice fields are sparse in the urban district, and mostly present in the distant rural districts of the province.
- The majority of the mosquitoes collected within Can Tho city belonged to three species of well known competent vectors for JEV: *Cx. tritaeniorhynchus*, *Cx. gelidus* and *Cx. quinquefasciatus*. Thus, both zoophilic and anthropophilic vectors can be present in all parts of the city, and found close to human dwellings.

- Presence of pigs in a household, as well as the number of pigs, was associated with an increase in the number of vectors. The number of vectors was highest close to the pigs, but pigs were also associated with an increase in the number of vectors close to the human dwellings. Seven mosquito pools collected close to pigs were found to be positive for JEV RNA, and six of these mosquito pools were from within Can Tho city.
- The nested RT-PCR established could detect JEV RNA in spiked mosquito pools, and dilution of mosquito samples 10-100 times is an effective way to avoid the PCR inhibition caused by mosquitoes.
- Within Can Tho city province the minimum JEV infection rate was approximately one per thousand mosquitoes. Viral RNA was detected in *Cx. tritaeniorhynchus* and in *Cx. quinquefasciatus*. This shows that JEV can be present in both a zoophilic vector and an anthropophilic potential bridge vector, which emphasizes the risk of JEV transmission to humans within the city. Both JEV genotype I and III could be detected in the same household in the city.

Taken together, these findings demonstrate that JEV is extensively transmitted in the Can Tho city province as well as in the city, indicating that JEV should not be regarded merely as a rural disease. Pig keeping can be an important feature in urban farming and this thesis shows an association with both increased numbers of vectors and the presence of JEV positive mosquitoes. It seems that pig keeping may increase the risks for humans to be exposed to JEV vectors and JEV, and the risk is even higher closer to the pigs. Pig keeping within an urban area may thus increase the risks for human infections.

6 Future perspectives

Japanese encephalitis virus has been recognized as a major human pathogen for almost a century. In spite of this, the virus keeps expanding its affected area, causing death and disabilities. There are still gaps in the general understanding of JEV epidemiology. It has not yet been convincingly shown how the virus keeps infesting temperate areas with non-continuous vector activity or how the virus is transmitted over large distances.

With growing urbanization the importance of urban animal keeping for vector-borne, as well as other zoonotic diseases, needs to be addressed. Although this thesis shows that transmission of JEV does occur within a city, several other questions concerning the epidemiology in a city remain. The breeding grounds within the city for the commonly rural vectors, such as the *Culex vishnui* subgroup, should be identified, and measures taken to decrease the presence of larval habitats. Since feeding preferences may differ between subpopulations of mosquitoes, blood meal analyses for the most common vectors in the city should be performed.

Pigs kept in urban areas are valuable for detection of transmission of JEV, and should be monitored in areas where it is common to keep pigs in urban farming. Dogs have also been proposed as good sentinels for the transmission of JEV to people, since they often live close to their owners. Studies on the seroprevalence in dogs in urban areas in Vietnam could therefore provide important information on the risk factors for JEV.

This thesis indicates that urban pig keeping may increase the number of JEV vectors to which humans are exposed. Taking measures to decrease the urban pig population may be one approach to minimize the risk of JEV transmission to humans. However, it must be remembered that urban pig keeping is an important contribution to the food supply within the city and to the livelihood of many households.

Some measures could be taken to decrease the infection pressure among the pigs and thus lower the viral load close to humans. Mosquito nets have been tested in an attempt to protect pigs from infection, but need to be maintained to really protect the pigs. If the infection pressure is reduced, there is, however, a risk that pig production could be negatively affected. Although vaccination of pigs within cities could be a possibility to decrease the JEV circulation, the constant high turnover makes vaccination cover costly. The most cost-effective way to diminish human cases is probably to ensure that all urban, as well as rural, inhabitants are protected by vaccination in the future.

Genotype I has been emerging in many parts of JEV-affected Asia and the co-circulation with genotype III in an urban mosquito population in South Vietnam may indicate that this shift is ongoing there as well. The presence of two genotypes increases the risk for recombination events to occur. To further understand the dynamics of the existing genotypes it would be valuable to isolate viruses and sequence whole genomes.

Other viruses with the potential to infect mammals probably circulate in the mosquitoes of the Mekong Delta of Vietnam, as well as mosquito-only flaviviruses. Increased screening of the mosquito population using general primers for alphaviruses, flaviviruses and bunyaviridae could provide more insight into this area, and the new techniques of metagenomics opens more possibilities for screening mosquitoes for viral infections.

The novel indication of the potential presence of JEV in Europe is alarming. If JEV starts transmitting among the mosquito and bird populations in Southern Europe, it could become wide-spread before clinical cases in humans appear, allowing JEV to strike against a virtually naïve population. The mosquito populations in countries, where JEV has not been reported yet, need to be monitored for their species composition, and their vector competence for JEV and other emerging arboviruses investigated.

In conclusion, several questions concerning the epidemiology of JEV and other arboviruses need to be addressed in the future, especially in the light of ongoing global trends.

7 Populärvetenskaplig sammanställning

7.1 Bakgrund

Japanskt encefalitvirus (JEV) är ett virus som är spritt i stora delar av södra och östra Asien. Viruset sprids mellan grisar och vadarfåglar med myggor i framförallt släktet *Culex*. Om en infekterad mygga råkar sticka en människa kan viruset orsaka Japansk encefalit, JE, en hjärninfection som har upp till 30% dödlighet och där hälften av överlevarna riskerar men för livet. Även hästar drabbas av dödlig hjärninfection, medan grisar sällan visar symtom, om de inte är dräktiga. Dräktiga grisar kan abortera, eller föda döda, mumifierade eller svaga kultingar.

Många av de myggarter som sprider viruset lägger ägg i risfält på landsbygden, där det finns gott om grisar och fåglar, och JE har huvudsakligen betraktats som ett landsbygdsproblem. Ökning av risodlingsarealen, ökad bevattning och ökad svinproduktion har lett till ökning av antalet JE fall i många länder, bland annat Vietnam. I norra Vietnam är klimatet tempererat och myggorna är aktiva under sommaren då det kan bli utbrott av JE, medan det i det tropiska södra Vietnam sker fall under hela året. I södra Vietnam, och särskilt i Mekongdeltaområdet, sker även huvuddelen av landets ris- och svinproduktion.

I den här avhandlingen studerades JEV i just Mekongdeltat. Först studerades hur vanlig JEV infektion är hos sugor i området runt Can Tho city och om det fanns något samband mellan genomgången infektion och antalet levandefödda kultingar. Därefter studerades myggor och grisar inne och runt Can Tho city för att se om det fanns ett samband mellan grisar inne i städer och antalet myggor samt om det fanns några bevis för spridning av JEV inne i själva staden.

7.2 Resultat och slutsatser

I den första studien konstaterades att det var svårt att se ett samband mellan genomgången infektion med JEV och nedsatt reproduktionsförmåga. Hos suggor under 1,5 år fanns ett samband mellan antikroppar mot JEV och fler dödfödda griskultingar. Anledningen till att det inte syntes ett samband med minskade levandefödda kultingar kan vara att de flesta gyltor smittas tidigt i livet, innan de blir dräktiga. Av samtliga 315 provtagna suggor hade totalt 60% antikroppar mot JEV.

I den andra studien studerades samband mellan grishållning i hushållen och antalet myggor som kan överföra JEV inne i själva Can Tho city. Ju fler grisar ett hushåll hade, desto fler myggor fanns där av två av de viktigaste myggarterna för spridning av JEV, *Culex tritaeniorhynchus* och *Culex gelidus*. Dessa myggor suger oftast blod från just lantbruksdjur. En tredje viktig myggart, *Culex quinquefasciatus*, var däremot opåverkad av grishållning men ökade i hushåll med många människor. *Culex quinquefasciatus* biter ofta människor men kan ibland även bita grisar och skulle därför kunna vara av stor betydelse för att sprida smittan från grisarna. Ingen myggart visade något samband med förekomst av risfält eller fiskdammar, vilket kan bero på att tillräckligt många andra platser där myggorna kan lägga ägg finns i städer, så att risfält inte är lika viktiga.

Alla grisar utom en som provtogs i den tredje studien hade antikroppar mot JEV, inklusive grisar som var födda inne i staden, vilket innebär att de måste ha smittats av JEV där. Flera grisar hade även visat symtom som skulle kunna varit orsakade av JEV, som aborter, omlöp, dödfödda eller svagfödda kultingar, eller kuling som darrat vid födseln. Eftersom det cirkulerar många virus i området som kan ge likadana symtom är det dock inte möjligt att säga vad som orsakat symtom i de här fallen.

Det gick att hitta JEV i sju prover som innehöll myggor, både prover som innehöll *Cx. tritaeniorhynchus* och *Cx. quinquefasciatus*. Två olika typer av JEV hittades, och det är första gången båda typerna har hittats i södra Vietnam och i samma stad. Sammantaget visar detta att bilden av JEV som ett landsbygdsproblem inte stämmer helt, utan att JEV kan bli ett ökande problem i städer i takt med ökad urbanisering och ökande behov av djurhållning inne i städer.

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¹³ For old times sake