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Seroprevalence of Japanese Encephalitis Virus in pigs and dogs in the Mekong Delta

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Seroprevalence of Japanese Encephalitis Virus in pigs and dogs in the Mekong Delta
Seroprevalens av Japanskt encefalitvirus hos grisar och hundar i Mekongdeltat

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LIST OF ABBREVATIONS

AES	Acute encephalitis syndrome
Arbovirus	Arthropod-borne virus
C	Capsid protein
Cx.	*Culex*
DENV	Dengue virus
E	Envelope protein
ELISA	Enzyme-linked immunosorbent assay
HI	Haemagglutination inhibition
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
PCR	Polymerase chain reaction
PrM	Pre-membrane protein
RT	Reverse transcriptase
SMEDI	Stillbirth, mummification, embryonic death and infertility
TBEV	Tick-borne encephalitis virus
WHO	World Health Organization
WNV	West Nile virus
YFV	Yellow fever virus

ABSTRACT

Japanese encephalitis virus (JEV) is one of the leading causes of acute encephalitis in humans. The virus is spread by mosquitoes, mainly belonging to the *Culex* species. The main reservoirs are considered to be birds and pigs, with pigs constituting the most important reservoir in regard to human infection. During the last few decades the production of pork has increased significantly in Vietnam. With 90% of pigs being kept in households owning 10 pigs or less, pigs are widely spread across most parts of the country. Due to the ongoing urbanization of Vietnam, the need for urban agriculture and animal husbandry has increased, resulting in an increasing amount of pigs/reservoirs being kept in urban environments. In the city of Can Tho, located in the Mekong delta, almost 100 % of the pigs have been found to be JEV positive, and vectors needed for transmission of JEV have been shown to be present in the urban areas of the city. The objectives of this study was to examine the seroprevalence of JEV in dogs and pigs in an endemic region, and to evaluate if dogs make suitable sentinels and could be used to evaluate the risk that JEV may pose to humans living in urban areas. To do this, blood samples were collected from dogs and pigs. A total number of 127 dog samples and 176 pig samples were collected from Can Tho city province and 65 samples were collected for comparative purposes from dogs originating from Ho Chi Minh City. The samples from Can Tho were divided into two equal parts. One part was frozen and sent to Sweden to be tested for JEV antibodies, using a competitive IgG ELISA. One part of the samples from Can Tho was also tested at Can Tho University using an in house HI-test. Due to denaturation as a result of high temperature inactivation of the sera, none of the samples that were sent to Sweden could be tested using the competitive IgG ELISA. The HI-results showed that 27 of the 127 dog samples and 159 of the 176 pig samples, all originating from Can Tho city province, had measurable antibody titres against JEV. Twenty three per cent of the dogs originating from the urban district of Ninh Kieu had measurable antibody titres against JEV. Although the seroprevalence was much higher among dogs mainly kept outdoors than dogs mainly kept indoors, it is notable that more than 16% of the dogs mainly kept indoors were also seropositive for JEV. These results indicate not only that JEV does pose a risk to people living in the urban areas of Can Tho city, but also that dogs may be good sentinels for human infection and can be used for evaluating the risk that JEV poses to humans living in urban areas.

SAMMANFATTNING

Japanskt encefalitvirus (JEV) är en av de främsta orsakerna till akut encefalit hos människor. Viruset sprids av myggor, framförallt myggor tillhörande arten *Culex*. De viktigaste reservoarerna är fåglar och grisar, där grisar utgör den viktigaste reservoaren i förhållande till humansmitta. Under de senaste årtiondena har produktionen av fläskkött i Vietnam ökat markant. Då mer än 90 % av grisarna hålls i hushåll med 10 grisar eller färre innebär detta att grisproduktionen är spridd över stora delar av landet. Till följd av den pågånde urbaniseringen av Vietnam har behovet av urbant jordbruk och djurhållning ökat, vilket har lett till en ökande andel grisar urbana miljöer. I Can Tho city, beläget i Mekongdeltat, har man funnit att nästan 100% av grisarna har antikroppar mot JEV samt att de vektorer som krävs för smittspridning förekommer i stadens urbana områden. Syftet med denna studie var att undersöka seroprevalensen av JEV hos hundar och grisar i en endemisk region, samt att utvärdera huruvida hundar är lämpliga som indikatordjur/sentineldjur och om de kan användas för att utvärdera den risk som JEV utgör för människor. För att undersöka detta samlades blodprover in från hundar och grisar. Totalt samlades 127 hundprover samt 176 grisprover in från Can Tho-regionen och i jämförande syfte samlades även 65 hundprover in från Ho Chi Minh City. Proverna från Can Tho delades upp i två lika delar och därefter frystes serumet. Ena halvan av varje prov skickades sedan till Sverige i syfte att testas för JEV-antikroppar med hjälp av en kompetetiv IgG-ELISA. Som resultat av högtemperatursbehandlingen, som gjordes för att inaktivera eventuella patogener i proverna, blev proverna som sändes till Sverige obrukbara och de kunde inte användas för en ELISA. Proverna från Can Tho testades på Can Tho universitetet med hjälp av deras egen HI-test. Resultaten från HI-testen visade att 27 hundprover och 159 grisprover var positiva för antikroppar mot JEV. Tjugotre procent av hundarna som härrörde från det urbana Ninh Kieu-distriktet hade mätbara antikroppstitrar mot JEV. Trots att seroprevalensen var signifikant högre bland hundar som till största delen hölls utomhus jämfört med hundar som till största delen hölls inomhus, så var en relativt hög andel av hundarna som hölls inomhus också seropositiva. Över 16% av hundarna i inomhusgruppen hade mätbara antikroppstitrar mot JEV. Resultaten tyder inte bara på att JEV utgör en risk för folk som lever i de urbana delarna av Can Tho city, utan också på att hundar möjligen kan användas såväl som sentineldjur som för att utvärdera den risk som JEV utgör för människor.

INTRODUCTION

Japanese encephalitis (JE) is a major cause of acute encephalitis syndrome (AES) in humans. Despite extensive immunization programs it has been estimated that almost 70 000 people will become ill with JE every year (Campbell *et al.* 2011). A majority of the cases affect children under the age of 15 (Zhang *et al.* 2008, Tan *et al.* 2010, Yen *et al.* 2010, Yin *et al.* 2010, Campbell *et al.* 2011). In up to one third of the cases the outcome is death, and many of the survivors will suffer crippling handicaps as a result of the disease (Buescher *et al.* 1959, Rosen 1986, Mackenzie *et al.* 2004, Kuno and Chang 2005).

The disease is caused by the Japanese encephalitis virus (JEV), a zoonotic arbovirus which is widespread in large parts of Asia (Solomon *et al.* 2003, Mackenzie *et al.* 2004). The virus, capable of infecting most vertebrates, is amplified mainly in pigs and wading birds and is spread by vectors (Buescher *et al.* 1959, Rosen 1986, Mackenzie *et al.* 2004, Kuno and Chang 2005). The main vector is *Culex tritaeniorhynchus*, a mosquito whose main breeding grounds are rice fields (Rosen 1986, Mackenzie *et al.* 2004, van den Hurk *et al.* 2009). Due to this the disease has been considered a rural disease and most immunization programs have mainly targeted children living in rural areas.

Because of the ongoing urbanization of many parts of Southeast Asia, urban agriculture and pig husbandry in cities are becoming more common (Van Veenhuizen and Danso 2007). Due to their potential to act as hosts for JEV, introduction of pigs into urban areas may result in an increasing risk for humans in these areas contracting JE. Recent research conducted in the Vietnamese city of Can Tho, located in the Mekong delta, has shown that both the virus and the vectors needed for transmitting JEV are present in the city (Thu *et al.* 2006, Lindahl 2012). It is therefore important to develop ways of evaluating the risk JEV may pose to humans living in cities where urban agriculture and pig husbandry are present. It is also important to find effective ways of monitoring the presence of JEV in cities. Pigs have often been used for JEV surveillance (Rosen 1986). However, a high percentage of pigs kept in areas where JEV is endemic are serologically positive (Lindahl 2012), which may make it difficult to evaluate risk factors for human exposure. Since pigs may be kept separate from human habitats, they may not accurately reflect the risk that JEV poses to humans living in cities.

Dogs can be infected by JEV, but do not develop symptoms nor pose a risk to humans since they do not become viraemic. Because of this, and of the way dogs are kept as pets and often live in the same habitat as humans, they may be useful as sentinels for JEV and for evaluating the risk JEV poses to people living in cities (Moore *et al.* 1993, Shimoda *et al.* 2010, 2011).

The purpose of this thesis was to assess the seroprevalence of JEV in dogs and pigs within the cities of Can Tho and Ho Chi Minh City and to evaluate if dogs are suitable as sentinels for JEV and can be used to assess the risk that JEV poses to humans living in urban areas in endemic regions, and which risk factors that are contributing to increased transmission.

LITERATURE OVERVIEW

The virus

The Japanese encephalitis virus (JEV) belongs to the family of Flaviviridae and the genus flaviviruses. This genus also contains several other viruses known to cause disease in humans, for example Dengue virus (DENV), Yellow fever virus (YFV), West Nile virus (WNV) and Tick-borne encephalitis virus (TBEV). Flaviviruses are small, single-stranded, enveloped RNA-viruses (Madrid and Porterfield 1974, Unni *et al.* 2011). Flaviviruses can be further divided into subgroups (sero-complexes or serological groups) based on serological cross-reactions. The Japanese encephalitis serological group include not only JEV, but also other mosquito-borne viruses such as WNV, St Louis encephalitis virus and Murray Valley virus (Madrid and Porterfield 1974).

Japanese encephalitis virus is divided into 5 genotypes, numbered I-V. The genotypes are based on nucleotide sequencing of the envelope protein (E) and premembrane protein (PrM), which together with the capsid protein (C) are genes coding for structural proteins of JEV (Unni *et al.* 2011).

Most of the flaviviruses use either mosquitoes or ticks as vectors and are therefore also called arthropod-borne viruses (arboviruses). Arboviruses can be further divided into mosquito-borne and tick-borne viruses. JEV belongs to the mosquito-borne viruses (Unni *et al.* 2011).

Epidemiology of Japanese Encephalitis

Flaviviruses are usually dependent on a vertebrate host or reservoir to allow the virus to replicate and spread. Japanese encephalitis virus is mainly maintained in a population through a biological transmission cycle where mosquitoes act as vectors, transmitting the virus between different host animals (Figure 1) (Kuno and Chang 2005). The main reservoirs are thought to be pigs, egrets, herons and other birds belonging to the family of Ardeidae, typically found around wetlands or bodies of water. Whereas birds are important maintenance hosts, pigs are considered to be of greater importance in respect to human infection (Mackenzie *et al.* 2004, Pan *et al.* 2011). The pig fulfils the main criteria for being an ideal reservoir; i.e. high reproduction rate, high turn-over rate, high infection rate, high viraemia lasting long enough to transmit the virus to another vector and a propensity for the vector to feed off it (Kuno and Chang 2005, van den Hurk *et al.* 2009). Humans and other animals such as horses, cats, and dogs, are so called accidental or dead-end hosts, and do not develop a viraemia high enough to pass the virus on to a new vector (Kuno 2001, Mackenzie *et al.* 2004).

As shown in Figure 2, JEV is spread all over southeast Asia reaching as far as Japan, India, the southern parts of Russia, and has also been found in Papua New Guinea and some parts of northern Australia. Different genotypes are present in different areas (Solomon *et al.* 2003, Mackenzie *et al.* 2004, Nga *et al.* 2004, Hills *et al.* 2011, Pan *et al.* 2011, Unni *et al.* 2011, WHO 2012).

The spread of JEV follows two different epidemiological patterns. In the temperate and sub-tropical regions the pattern is usually of an epidemic nature, with outbreaks of JE occurring mainly in summer or early autumn, just after the rainy season. In regions with a more tropical climate the pattern is mainly endemic (Halstead and Jacobson 2003, Mackenzie *et al.* 2004, van den Hurk *et al.* 2009).

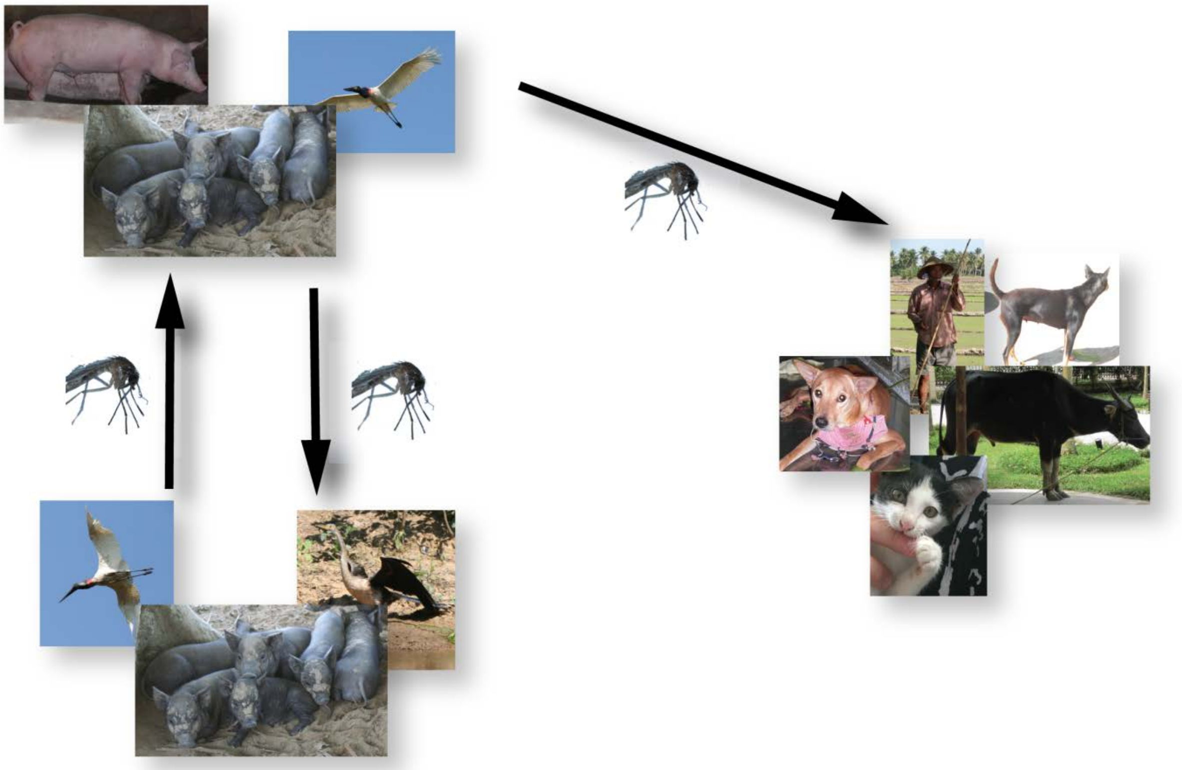

Figure 1. Transmission cycle of JEV.

Vietnam and JEV

The first human JE case in Vietnam was reported in the beginning of the 1960s (Erlanger *et al.* 2009), and the first outbreak of JE was reported in the mid 1960s (Igarashi and Takagi 1992). Since then, JEV has been present in the country and has been shown to be the cause of more than 50% of all cases of acute encephalitis syndrome (AES) in Vietnam (Yen *et al.* 2010). An annual incidence of 1000-3000 cases is expected, most of them affecting children under the age of 15 (Lowry *et al.* 1998, Erlanger *et al.* 2009, Yen *et al.* 2010).

A national immunization program against JE was initiated in 1997. In the initial stage only school children in high risk areas were immunized against JE, but by 2007 the program had expanded to include Vietnamese children in approximately 65% of the districts (Yen *et al.* 2010). However, in a study by Tan *et al.* (2010) only 19.5% of the children presenting with clinical signs of AES, who were admitted to a referral hospital in Ho Chi Minh City in 2004, were reported to have previously gotten at least one dose of JEV vaccine.

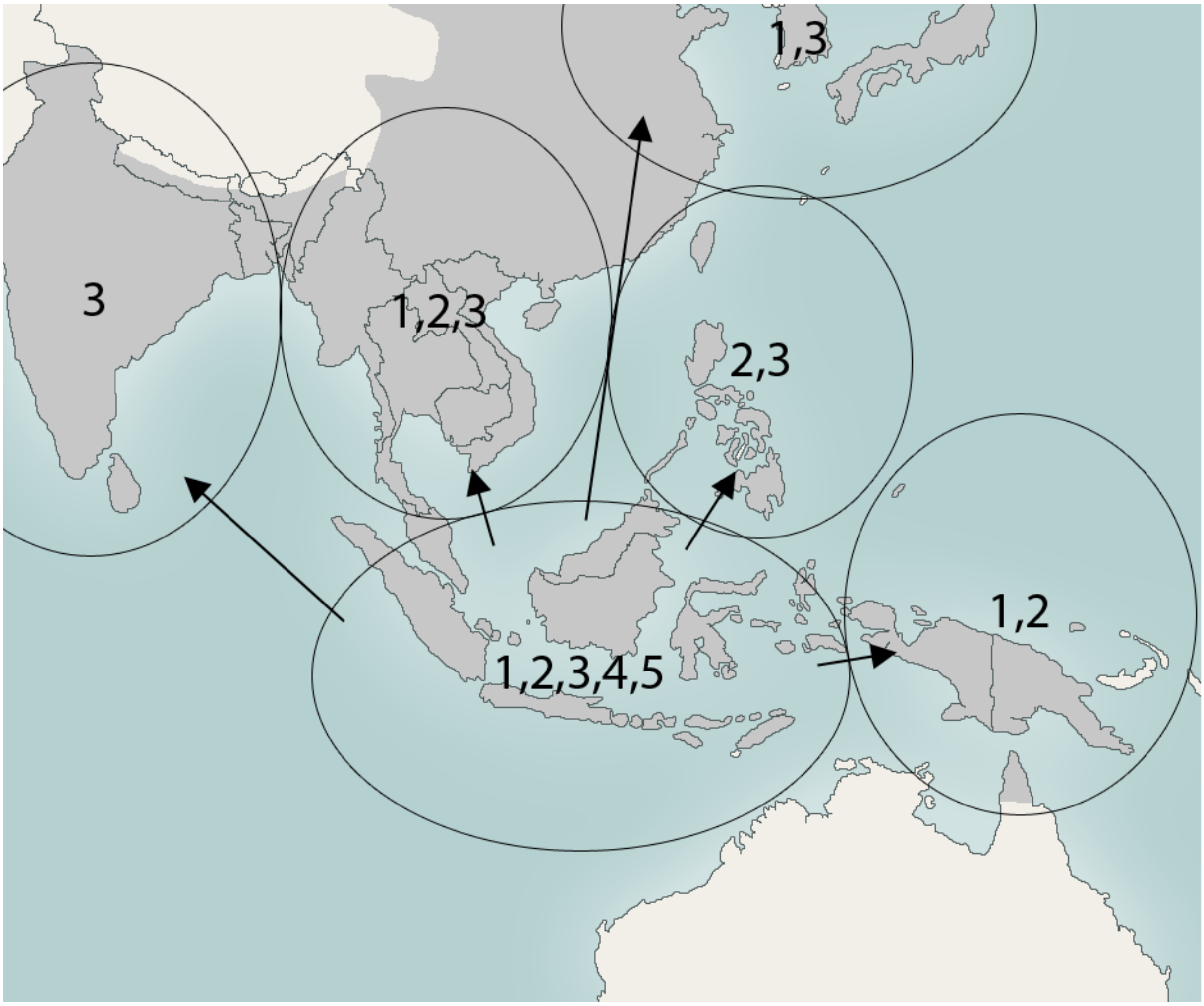

Figure 2. Map showing the distribution of JEV and spread of JEV genotypes. Grey areas show the areas where JEV has been reported. Numbers represent the genotype present in the encircled areas. (Based on Solomon et al. 2003, Mackenzie et al. 2004, Hills et al. 2011. Map modified from © OpenStreetMaps.org contributor.)

In the temperate northern parts of Vietnam, JEV is considered to be of an epidemic character and the number of cases usually peaks in the summer months, while in the tropical southern parts of Vietnam, JEV is endemic and with the occurrence of cases being more evenly distributed over the year (Do *et al.* 1994, Lowry *et al.* 1998, Yen *et al.* 2010).

In a report by Yen *et al.* (2010) covering 1998-2007, it was found that although the number of AES cases in Vietnam were overall declining, the incidence of AES still remained high in some of the main rice-growing areas around the Red River delta in the north and around the Mekong delta in the south. Factors such as increasing pig production and the increase in areas used for rice production, might be contributing to the emergence of JE in Vietnam (Solomon *et al.* 2000, Erlanger *et al.* 2009).

In the 1950s, only 10% of the population in Vietnam lived in urban areas. Since then the urban population has grown even more, increasing from 20.3% in 1999 to 29.6% in 2009. In some parts of Vietnam an excessive urbanization has led to poverty and unemployment (MPIGSO 2011). Due to this, many urban households use urban agriculture and urban animal husbandry as a source of income, and if keeping animals in addition to agriculture, pigs are often the preferred choice (Schiere and Hoek 2001, van Veenhuizen and Danso 2007). The pork production of Vietnam has increased rapidly and between 1995 and 2006, the increase in the number of pigs was nearly 65%, or ten million pigs. In 2010 it was reported that there were more than 27 million pigs in Vietnam (GSO 2012b). Almost 20% of the pork production originate from the Mekong area (Tisdell 2008). Ninety per cent of the pigs are kept in households owning 10 pigs or less (Tisdell 2008).

In Can Tho city province, JEV vectors have been found to be present in the urban as well as rural districts and almost 100% of the pigs can be JEV positive (Thu *et al.* 2006, Lindahl *et al.* 2012b).

Humans and JEV

During 2006-2009 an average of 6075 human cases of JE were annually reported to the World Health Organization (WHO). This number probably represents an underestimate of the actual number of cases, which may be more than ten times higher. Difficulties in diagnosing JE and limitations of some health care establishments in developing countries make it hard to confirm the diagnosis of JE and differ it from other causes of AES. Furthermore health statistics from many countries are lacking or may be incomplete (Campbell *et al.* 2011).

Most JEV infections occurring in humans are subclinical and do not result in disease. Southam (1956) found that for every clinical case there were up to 1000 subclinical cases. However, much lower ratios (1:25) have also been seen when JEV is introduced into a population formerly naïve to the virus (Halstead and Grosz 1962).

In humans, JEV causes a neurotropic viral infection with meningoencephalomyelitis. The virus uses leukocytes to reach the CNS, and is internalized into the CNS by means of endocytosis (Ghosh and Basu 2009). The most commonly observed clinical manifestation of JE is acute encephalitis. The clinical signs of acute encephalitis include fever, severe headache, nausea, vomiting, changes in mental status such as confusion, disorientation, coma, or inability to talk, and seizures (Lincoln AF 1952, Solomon *et al.* 2000, Hills *et al.* 2009). Another symptom sometimes seen in cases of JE is flaccid paralysis. Especially in children, flaccid limb paralysis might be a prominent clinical sign, and sometimes even the only clinical sign observed (Solomon *et al.* 1998, 2008). The incubation period after a mosquito bite ranges from 3 to 15 days, but longer incubation periods may also be possible (Tiroumourougane *et al.* 2002, CDC 2013, SMI 2013).

Case fatality rates range from only a few per cent to up to 60%, although rates in the span of 15-30% are more often reported (Solomon *et al.* 2002, Halstead and Jacobson 2003, Bista and Shrestha 2005, Maha *et al.* 2009, Yen *et al.* 2010). Since there are no specific antiviral drugs against JE, treatment is mainly focused on relieving symptoms and on supportive care (Tiroumourougane *et al.* 2002, CDC 2013).

More than 50% of the patients surviving the clinical disease suffer from moderate to severe sequelae in the form of tremors, seizures, paralysis and abnormalities in mental function, such as amnesia, memory defects, poor orientation, poor attention span, and lower IQ. The proportion of sequelae is especially high in children under the age of 15 (Ketel and Ognibene 1971, Solomon *et al.* 1998, 2000, 2002, Ding *et al.* 2007, Maha *et al.* 2009).

Pigs and JEV

Pigs are important reservoirs for JEV, especially in regard to human JEV infection (Rosen 1986, Gubler 2002). The presence of pigs in a region is associated with increased risk for humans and horses contracting JE (Gubler 2002).

Two to three days after infection, primarily infected pigs normally develop a viraemia lasting for a few days (Scherer *et al.* 1959, Williams *et al.* 2001). However, it has also been shown that young piglets can develop viraemias lasting for as long as two weeks (Nitatpattana *et al.* 2011). It is during this period that JEV may be transferred to a mosquito vector feeding off the viraemic host, and in that way spread to other vertebrates.

As a result of JEV infection the sow or gilt produces antibodies, which can be passed to the newborn, suckling piglets via the colostrum. These maternal antibodies provide the piglets with protection against JEV infection, for a period lasting between 3-6 months (Scherer *et al.* 1959, Nitatpattana *et al.* 2011).

Clinical signs like fever, malaise, and tremors have been observed in pigs infected with JEV, but are considered to be fairly unusual. More common symptoms of infection are reproductive disturbances such as still-birth, mummification, embryonic death, and infertility (also known as SMEDI syndrome) (Burns 1950, Hosoya *et al.* 1950, Shimizu *et al.* 1954, Morimoto *et al.* 1972, Gresham 2003, Daniel Givens and Marley 2008). In epidemic areas, high rates of SMEDI symptoms have been reported in pigs during breakouts of JE in humans and horses (Burns 1950, Hosoya *et al.* 1950). Abortion is rarely seen in cases of JEV infection (Gresham 2003, Epiwebb 2012).

Reproductive disturbances seem to be more usual in areas where the spread of JEV follows an epidemic pattern, than in areas where the virus is endemic (Morimoto *et al.* 1972, Lindahl 2012). In endemic areas, where the infection pressure is high, many gilts will probably have been infected with JEV at an early age and have acquired a protective immunity before being mated for the first time (Lindahl *et al.* 2012a, Lindahl 2012). Lindahl *et al.* (2012a) found no evidence of JEV-positive sows having a higher rate of still-born piglets than JEV-negative sows when researching this in an endemic area in the Mekong delta. It is thus likely that the reproductive disturbances seen in pigs as a consequence of JEV infection, is of little importance in areas where JEV is endemic.

Foetuses may be affected if the sows or gilts contract the primary infection around the time of mating or during the first 60-70 days of gestation after which the foetuses become more resistant to infection (Gresham 2003, Daniel Givens and Marley 2008). Weak-borne piglets may be born with hydrocephalus and subcutaneous oedema (Burns 1950, Hosoya *et al.* 1950, Gresham 2003, Daniel Givens and Marley 2008).

Due to JEV infection boars may suffer from infertility or decreased sperm count as a result of febrile episodes caused by the virus. The virus can be shed in the sperm and can thus infect naïve sows or gilts (Habu *et al.* 1977, Gresham 2003, Maes *et al.* 2008).

Dogs and JEV

Dogs can be infected with JEV, but do not develop a viraemia and usually seroconvert without showing any, or only few, clinical signs (Shimoda *et al.* 2011). Shimoda *et al*. (2011) found that dogs infected with JEV genotype III had rising antibody titres during the first 3-4 weeks. Antibody titres remained high until 70 days post infection. The dogs did not express any clinical symptoms except for a mild weight loss with duration of 1-2 days. Blood analyses showed a temporary rise in C-reactive protein during the first few days after inoculation (Shimoda *et al.* 2011).

Birds, other animals and JEV

Although wild, water-living birds belonging to the family of Ardeidae are considered to be the main maintenance reservoirs, other wild birds as well as domestic birds such as chickens and ducks can also be infected with the virus. As a result of infection they can develop a viraemia, but whether they are to be considered important reservoirs remains unclear (Buescher *et al.* 1959, Scherer *et al.* 1959, Johnsen *et al.* 1974, Rosen 1986).

Different species of bats can carry the virus and may be important as host animals. Unlike other vertebrates, bats may also become infected by ingesting vectors carrying the virus (Kuno 2001, Kuno and Chang 2005).

Other animals, such as buffalo and cattle, can be infected with JEV, although these species rarely seem to develop a viraemia high enough to transfer the virus to mosquitoes (Ilkal *et al.* 1988, Mwandawiro *et al.* 2000, Arunachalam *et al.* 2005). Cattle might even be a kind of zooprophylaxis, because they prevent mosquitoes from biting humans and pigs (Mwandawiro *et al.* 2000, Arunachalam *et al.* 2005). Since mosquitoes prefer to feed on the same animal species as they previously fed on, this may enhance the zooprophylactic effect of cattle (Johnsen *et al.* 1974, Rosen 1986, Mwandawiro *et al.* 2000, Arunachalam *et al.* 2005).

Horses may sometimes develop encephalitis as a result of JEV infection. Infection is however often subclinical, or cause only mild clinical signs such as fever (Gould *et al.* 1964, Rosen 1986, Epiwebb 2012).

Vectors

As implied by the name, most arboviruses are dependent on a biological vector for transmission (Kuno and Chang 2005, Unni *et al.* 2011). Japanese encephalitis virus is mainly transmitted by mosquitoes belonging to the *Culex* species, especially the *Culex vishnui* subgroup. *Culex tritaeniorhynchus*, in the *Cx. vishnui* subgroup, is considered to be the most important vector for JEV transmission (Mackenzie *et al.* 2004). The main breeding grounds for the *Cx. vishnui* subgroup are considered to be rice fields (Reuben 1971, Sunish and Reuben 2001). However, many other mosquitoes, both in other subgroups of the *Culex spp.*, and mosquitoes belonging to other genera such as *Aedes*, *Anopheles,* and *Mansonia*, are capable of transmitting the virus and may also play an important role in JEV transmission (Rosen 1986).

Feeding behaviour of mosquitoes

Mosquitoes are opportunistic feeders, and their feeding preferences depend on density and availability of vertebrate hosts (Kuno and Chang 2005). *Culex tritaeniorhynchus* is zoophilic and prefers to feed off animals rather than humans. In areas where there is a high cattle population, *Cx. tritaeniorhynchus* seem to prefer to feed off cattle while in areas with a dense pig population it feeds off pigs to a higher extent, thus confirming its opportunistic feeding pattern (Reuben *et al.* 1992, Mwandawiro *et al.* 2000, Arunachalam *et al.* 2005). When tested for ingested blood meals, mosquitoes belonging to *Cx. tritaeniorhynchus* often contain blood from more than one animal species, indicating that they often feed off several different hosts. This behaviour may increase the risk of virus transmission to other hosts (Arunachalam *et al.* 2005, Samuel *et al.* 2008).

Culex pseudovishnui, *Cx. vishnui* and *Culex gelidus* are all important, mainly zoophilic, vectors, while *Culex quinquefasciatus* is considered to be an important human vector because it often feeds on humans, i.e. it is considered to be an anthropophilic vector (Colless 1959, Reuben *et al.* 1992). In many studies *Cx. quinquefasciatus* has also been found to have fed to a high extent on birds and dogs, with reports of 5-14% of the blood meals originating from dogs (Colless 1959, Mitchell *et al.* 1973, Reuben *et al.* 1992, Garcia-Rejon *et al.* 2010, Alencar *et al.* 2012).

Detecting JEV

Virus detection

Virus detection can be done by virus isolation or by molecular methods, such as reverse transcriptase (RT) polymerase chain reaction (PCR). These methods can be used to monitor and detect JEV in blood samples taken from viraemic individuals and also to detect virus present in vectors (Williams *et al.* 2001, Hall *et al.* 2012).

Virus isolation is often used in JEV surveillance. Because virus is only possible to isolate during a short period of time, the method may be of limited use in the field (Williams *et al.* 2001, Johansen *et al.* 2002, Hall *et al.* 2012). While the virus is detectable for about 2-3 days during a viraemic period in primarily infected pigs, it might not be detectable at all in pigs that have formerly been infected with JEV or other flaviviruses such as dengue virus or WNV (Williams *et al.* 2001). Virus isolation involves inoculating for example mice, embryonated eggs, or vertebrate cells with live virus. Some of the drawbacks of this procedure are that it is time consuming and demands the isolation and handling of viable virus (Hall *et al.* 2012).

Molecular methods such as PCR and reverse transcriptase PCR (RT-PCR) detect viral DNA and RNA respectively. These methods are considered more rapid, more sensitive, and do not demand the handling of viable virus (Williams *et al.* 2001, Hall *et al.* 2012). If correctly stored, viral RNA may remain relatively stable for up to 2 weeks (Johansen *et al.* 2002). Thus RT-PCR might be the preferred method when analyzing mosquitoes for the presence JEV.

Serological methods

Two common serological methods are the haemagglutination inhibition test (HI) and the enzyme-linked immunosorbent assays (ELISA). Both of these methods can be used to detect IgG and IgM antibodies in serum or cerebrospinal fluid (CSF) taken from suspected host animals or infected individuals (Ishii *et al.* 1968, Madrid and Porterfield 1974, Hall *et al.* 2012). One of the drawbacks of serological testing is the risk of cross-reactions between different flaviviruses, especially those belonging to the same serological group. In particular JE and WNV have a tendency to cross-react (Madrid and Porterfield 1974, Kimura-Kuroda and Yasui 1986, Hall *et al.* 2012). The HI method is a relatively cheap method, but there is an even higher risk for cross-reactions between different flaviviruses such as JEV and dengue virus with this method than with an ELISA (Madrid and Porterfield 1974, Kimura-Kuroda and Yasui 1986, Kuno 2003, Robinson *et al.* 2010).

In pigs, IgM antibodies are detectable within two to three days of infection and usually decrease rapidly after about two to three weeks, while IgG antibodies are detectable at the earliest approximately one week post infection, but may be detectable for a longer period than IgM antibodies (Ishii *et al.* 1968, Burke *et al.* 1985).

JEV control and JE prevention

There are basically three ways of preventing and controlling the spread of arboviruses like JEV; reduction of vectors and their habitats, reduction of amplifying hosts/reservoirs, and through different measures to protect individuals from being infected (Tiroumourougane *et al.* 2002, Saxena and Dhole 2008, Dutta *et al.* 2010).

Vector control

Vector reduction or vector control techniques include for example spraying of mosquito habitats using an appropriate insecticides or larvicides, thermal fogging (using very low concentrations of insecticides) of local areas, irrigation- and flooding techniques, or planting fish in ponds to control the larvae population (Tiroumourougane *et al.* 2002, Keiser *et al.* 2005, Saxena and Dhole 2008). The methods may be temporarily effective, but due to the vast and varying mosquito dwellings, it is not possible to control the spread of JEV solely by using these types of methods (Eldridge 1987, Tiroumourougane *et al.* 2002, Dutta *et al.* 2010, Shaman 2011).

Reservoir control

Measures taken to reduce the number of reservoirs are preferably directed at domestic pigs and pig farming rather than at wild pigs and birds, because the latter are hard to control. Industrialization of pig rearing as well as relocation of pig farms to areas segregated from human habitats may be effective ways of reducing the risk of humans being bitten by infected mosquitoes, but do not completely eliminate the risk for humans getting infected with JEV (van den Hurk *et al.* 2001, van den Hurk *et al.* 2008, Ting *et al.* 2004, Oya and Kurane 2007). These types of measures may not be possible in poor rural areas, where farmers own only a few pigs and are depending on them for their livelihood.

Another approach focusing on the pig as reservoir is vaccination of pigs, which result in fewer SMEDI symptoms as well as a lower rate of infection. Vaccination does, however, not provide 100% protection (Van den Hurk *et al.* 2009). Due to the high turnover in the pig industry, this alternative is expensive and may be inefficient in protecting humans against JEV infection (Grossman *et al.* 1974).

In poor rural areas the use of a mosquito net impregnated with insecticide being kept over pig pens have been seen to reduce the number of infected pigs as well as reducing the JEV transmission to humans. This approach may, however, cause pigs to get infected later in life and thus increase the risk for reproductive disturbances in the pigs (Dutta *et al.* 2011).

Preventing infection

Vaccination is one of the main ways of preventing JE in humans. It is generally recommended for people living in endemic areas, and travellers visiting areas where JEV is endemic for more than 30 days or travellers visiting areas where there is an ongoing epidemic (Tiroumourougane *et al.* 2002, Dutta *et al.* 2010).

In Japan, massive vaccination campaigns have been successful in greatly reducing the number of JE cases, although not completely eradicating JEV (Arai *et al.* 2008). Today there are several different vaccines being used, of which only one is approved by the WHO (Saxena and Dhole 2008). The vaccines have an efficacy of between 85 – 95% (Tiroumourougane *et al.* 2002, Saxena and Dhole 2008). Since some of the vaccines can cause allergic reactions, do not give a long term protection and are expensive to produce, they might not be practical for vaccination of people in poor, rural areas. Cheaper and more effective vaccines are needed (Mackenzie *et al.* 2004, Saxena and Dhole 2008). Research into DNA vaccines, peptide-based vaccines and different recombinant vaccines is in progress (Saxena and Dhole 2008, Dutta *et al.* 2010).

Other prophylactic methods like using long sleeved shirts and trousers, sleeping indoors, using insecticide-treated mosquito nets, and using screen doors and windows have been shown to be effective in reducing the risk of being bitten by mosquitoes, thus reducing the risk of being infected with JEV (Luo *et al.* 1994, Tiroumourougane *et al.* 2002, Dutta *et al.* 2011).

Dogs as sentinels

The use of animals as sentinels to evaluate different risk factors to humans is not new. One example of this use of animals is the canary bird kept in coal mines to alert the miners of rising levels of carbon monoxide.

To be considered a good, or ideal, candidate for being used as a sentinel the individual animal should meet the following requirements (Moore *et al.* 1993):

- Live close to humans, preferably in the same habitat/dwelling as humans.
- Be susceptible to the infection through the same means of transmission that are used to transmit the virus to humans.
- Be able to survive the infection and preferably not get sick.
- Develop antibodies in an amount that is easy to detect and sustain the antibody titre over a longer period of time.
- Not develop a viraemia with virus titres high enough to be able to transmit the virus to mosquitoes and thus pose a risk to humans.
- Preferably attract the same vector species as humans attract.
- Should be easy to handle and sample.

Dogs also have a shorter life span than humans and may sometimes develop disease more rapidly (Committee on Animals as Monitors of Environmental Hazards, Board on Environmental Studies and Toxicology, National Research Council 1991, Backer *et al.* 2001, Komar *et al.* 2001, Kile *et al.* 2005). Dogs have previously been shown to be useful as sentinels not only when it comes to environmental hazards but also as sentinels for some arboviral diseases such as West Nile fever and JE (Backer *et al.* 2001, Komar *et al.* 2001, Kile *et al.* 2005, Shimoda *et al.* 2010, 2011, 2013, Reif 2011).

MATERIALS AND METHODS

Study area

Can Tho city province

Can Tho city province (Thành phố Cần Thơ) is situated in a tropical region in the southern part of Vietnam (Figure 4). It is the largest city of the Mekong delta and the fifth largest city in Vietnam. The total population of Can Tho city province is estimated to 1.2 million people and the province covers an area of 1409 km^2, resulting in an average population density of 852 persons/km^2 (2011). The urban population is estimated to almost 800 000 (791 800) people (GSO 2012a, SOCTC 2012).

Can Tho city province is divided into nine districts, which are further divided into wards. The districts are mainly rural and focused on agriculture and animal husbandry. In a majority of the districts large areas are covered by rice paddies and pigs are often kept in close vicinity to human habitats. In 2010, more than 121 000 pigs were reported in Can Tho city province (SOCTC 2012).

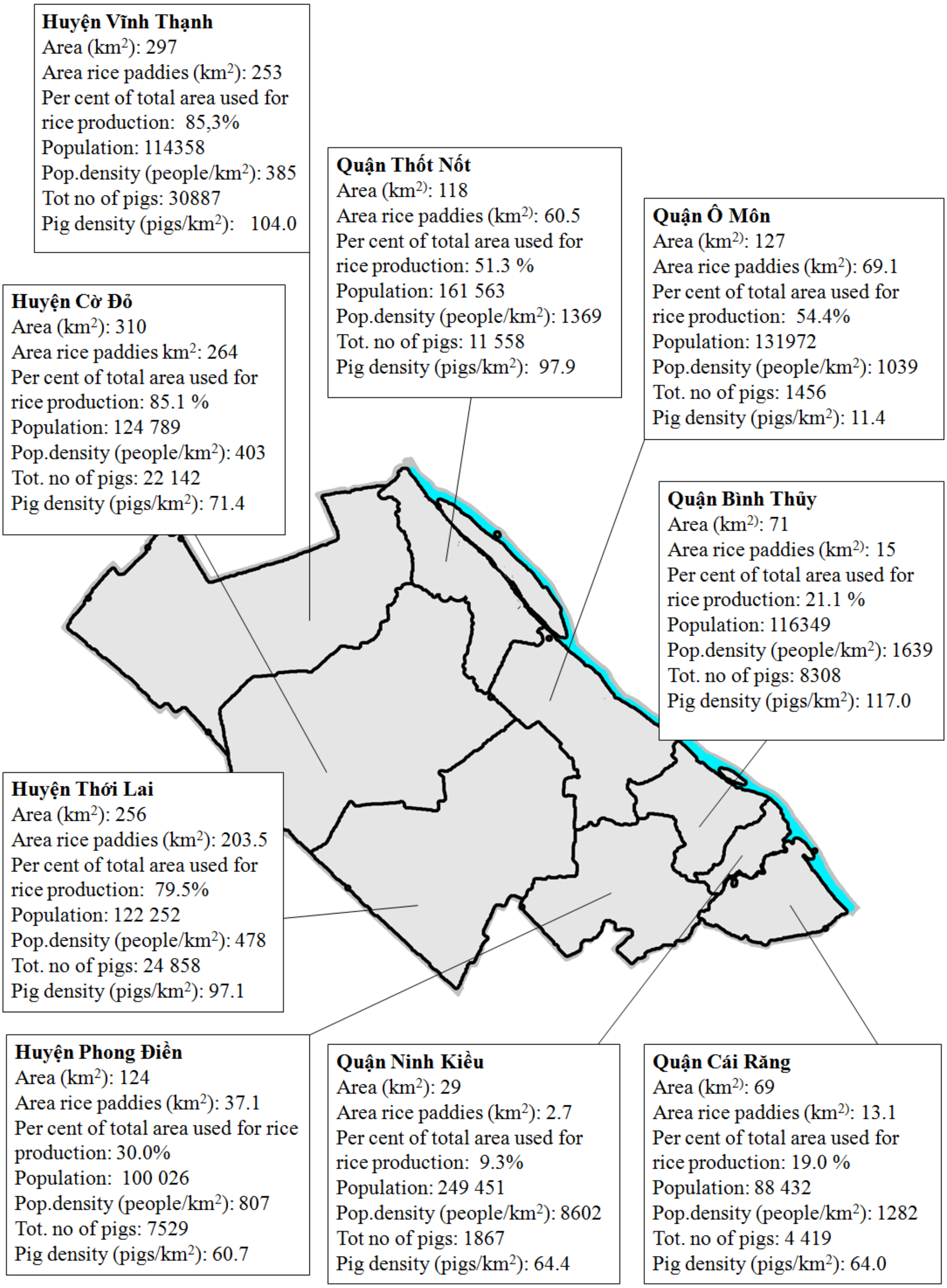

Figure 3. Statistical data for the districts of Can Tho city province (GSO 2012a, SOCTC 2012).

In contrast to the rural areas, the Ninh Kieu district (Quận Ninh Kiều) is an urban and well populated district, with a population of almost 250 000 people, resulting in a population density of 8600 people/km^2 (GSO 2012a). The Ninh Kieu district is subdivided into 13 wards.

The total number of officially reported pigs in Ninh Kieu 2010 was 2600 (GSO 2012a, SOCTC 2012). Statistical data for the different districts is shown in Figure 3.

During the wet season lasting from May until October/November, Can Tho is intermittently flooded due to its low topography in combination with high tide and rainfall occurring in short and intense periods. Since the drainage system is incomplete without enough capacity, flooding often results in pools of stagnant water within the city. These pools usually do not completely dry out until after the end of the flooding season (Huong and Pathirana 2011).

Ho Chi Minh City

Ho Chi Minh City (Thành phố Hồ Chí Minh), formerly known as Saigon, is located in the south eastern part of Vietnam, and situated on the Saigon river. It is the largest city in Vietnam, with more than 7.5 million people living in the city alone. The city covers almost 2100 km^2 in total - the urban area consisting of 19 districts and counties covering 140 km^2, and the rural areas comprising 5 sub-districts with 98 communes covering almost 1 900 km^2. The total population density is almost 3600 people/km^2. In 2009 almost 6 million of the inhabitants were living in the urban area, resulting in an urban population density of almost 43 000 people/km^2 (GSO 2012a, DPI 2008, MPIGSO 2011). Rice paddies are not present within the city and keeping of pigs is prohibited within the city limits.

Study population and study design

Sample collection

Pigs

Blood samples from pigs were collected at one of the local slaughterhouses located in the Ninh Kieu district, in Can Tho city during a three week period, between October and November 2012. Samples were collected at night in connection with the de-blooding of animals, using a serum vial to collect the blood. The blood was allowed to settle at room temperature for approximately 30 minutes before being put in a cooler with ice. Samples were brought to the laboratory, centrifuged and serum was separated and divided into two equal parts. The samples were then stored in small tubes at -18ºC until transportation to Sweden or HI analysis was performed. Data concerning origin, breed, sex, weight and age of the animals was collected in connection with the sample collection.

A total of 192 blood samples from pigs were collected. Out of 192 samples, 176 samples were taken from pigs originating from Can Tho city province. The 176 samples were fairly evenly distributed over 7 out of 9 districts with the number of samples per district ranging from 22 – 28. The exact distribution is shown in Figure 5 and Table 2. No samples were acquired from the urban district of Ninh Kieu or from the Binh Thuy district (Quận Bình Thủy). Samples from pigs not originating from Can Tho city province (n=18) were excluded from this research.

Dogs

During an eight week period between September and November 2012, blood samples were collected from dogs visiting the Can Tho University Veterinary Clinic after consent from the owners. Blood was drawn from a vein in one of the front legs, using a canula and syringe. The blood was then transferred to a serum vial, which was left to settle at room temperature (approximately 25-30ºC) for 30-60 minutes, after which the vial was stored in a cooler filled with ice for a few hours. Every afternoon the samples were collected from the veterinary clinic and taken to a laboratory, where the samples were centrifuged and the serum separated. The samples were then handled as the swine serum samples. The owners were asked to complete a questionnaire with questions regarding their pets age, breed, sex, whether their dog were mainly kept indoors or outdoors, address, and if there was stale water or pigs in the vicinity of the dogs' habitat. The only inclusion criteria used was that the dog should be older than 6 months of age. Clinical illness was not an excluding factor. No recordings were made of whether the sample was collected from a healthy dog only coming in for a vaccination, or from a dog showing clinical signs of disease.

During a two week period in October, blood samples from dogs were collected at a local dog slaughter house in Can Tho city. The procedure was the same as for collection of the blood samples from pigs described above.

During a two week period between October and November, blood samples were collected from dogs visiting the Saigon Pet Clinic, located in Ho Chi Minh City. The sample collection and collection of data were performed using the same procedure used at Can Tho University Veterinary Clinic, with the exception of the separated sera not being divided into two equal parts and not being analysed using HI.

A total of 88 dog samples were collected at the Can Tho University Veterinary Clinic and 56 samples from the local dog slaughter house in Can Tho, making a total of 144 samples. Out of the 144 dogs, 127 originated from the districts of Ninh Kieu, O Mon (Quận Ô Môn), Cai Rang (Quận Cái Răng) and Thot Not (Quận Thốt Nốt), with the number of samples per district ranging from 1-75. The exact distribution is shown in Figure 6 and Table 4. Samples from dogs not originating from Can Tho city province (n=17) were excluded from this research.

Sixty five samples of dog sera were collected at the clinic in Ho Chi Minh City. The collected data concerning these samples is not presented in this thesis.

Questionnaire

During an eight week period, random tourists, expatriates and local Vietnamese were asked to complete a questionnaire concerning their awareness of JEV. Depending on nationality, people were asked to either complete a questionnaire in English or one in Vietnamese. Questions concerning age, sex, occupation, vaccination status, having heard of JE and JEV, concerning how JEV was spread and precautions taken to avoid mosquitoes were common to both questionnaires. The English questionnaire, targeted at tourists and expatriates, also contained questions regarding the reason for visiting Vietnam, the length of their stay and their accommodation while in the country. The Vietnamese questionnaire, targeting local Vietnamese people, in addition to the common questions, also addressed whether they owned dogs or pigs or lived near rice fields, stagnant water or pigs. The questionnaires were handed out in Can Tho, Mui Ne and Rach Gia, located as shown in Figure 4.

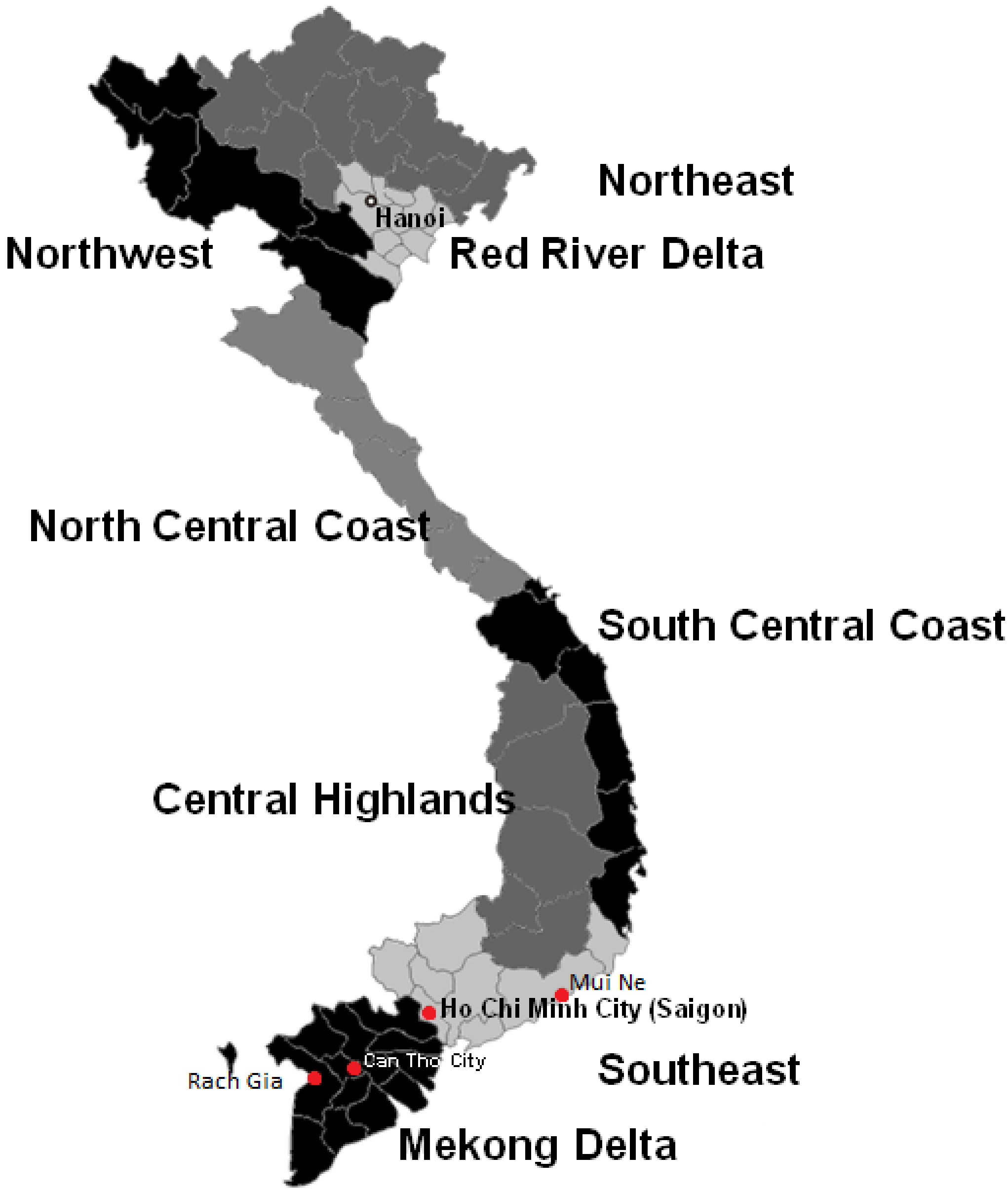

Figure 4. Showing a map of Vietnam. The red dots represent the locations were blood samples were collected (Can Tho city and Ho Chi Minh City) or questionnaires were handed out (Rach Gia, Can Tho city and Mui Ne) (Map modified from Lindahl 2012).

Serological analyses

Preparation

After collection, samples were stored at Can Tho University at -18 ºC until transport to Sweden. The samples were then transported in a frozen state, in an ice box with ice packs, to SVA in Uppsala, Sweden, by use of courier. Samples were inactivated by heating them to

72ºC for two hours, due to regulations at the lab assigned to inactivate the samples, causing coagulation of the samples.

Control procedure

Due to the serum being coagulated, a trial inactivation and ELISA was conducted first. A positive control of pig serum and some known negative pig sera was inactivated using the same temperature and duration as had been used to inactivate the collected samples of dog and pig sera. This resulted in denaturation of all the samples, and the sera forming coagulas with no or very little liquid suspension. The serum coagula were mechanically separated using a pipette and was suspended in equal amounts PBST for 24 hours to extract antibodies from the sera. The two known pig samples and 14 samples collected from dogs were then tested using the competitive IgG ELISA described below.

ELISA

A competitive IgG ELISA was attempted, using antigen, antibodies, conjugate and controls provided from Australian Animal Health Laboratories (AAHL, Geelong, Victoria, Australia), according to previously described procedures (Williams *et al.* 2001, Pant *et al.* 2006).

The 96-well microplates were prepared by coating wells with 50µl with JEV antigen diluted in Carbonate buffer. Following overnight storage at 4ºC, the wells were washed with PBS-TWEEN (PBST). Serum was diluted 1:10 in PBST + 1% skim milk powder. Fifty µl of diluted test serum was added to each well. Positive control serum was diluted in the following dilutions: 1/100, 1/800, 1/1000 and 1/2000. For the negative control, negative pig serum in a 1/10 dilution was used. Plates were then incubated in 37ºC on a plate shaker for 1 hour. Fifty µl of monoclonal antibody (diluted 1/4000) was then added to all wells except for the two controls. After renewed incubation at 37ºC for 30 minutes and washing, HRPO conjugate in 1/2500 dilution was added. Plates were incubated at 37ºC on a shaker and washed before addition of 50µl of TMB (3,3´,5,5´-Tetramethyl-benzidine, SVANOVA, Uppsala, Sweden). After incubation at room temperature for 5 minutes, reaction was stopped by adding 50µl of 1M H_2SO_4 to each well. The plates were read at 450 nm.

According to the instructions, the levels of inhibition were calculated and samples resulting in levels of less than 40% inhibition were interpreted as negative and levels greater than 50% inhibition were interpreted as positive. Levels between 40-50% were considered inconclusive.

HI testing

HI testing of the samples collected in Can Tho city province, was performed at Can Tho University according to their in-house laboratory methods (Thu *et al.* 2006b). The samples from Ho Chi Minh City were not subject to HI testing.

Statistics

Statistics were performed using PASW Statistics 18, SPSS Inc. Chi-square test and Fisher's exact test were used to evaluate the correlation between age and vaccination status, having heard of JE and use of repellent, and the correlation between nationality (defined as others and Vietnamese) and having heard of JE. A T-test was used to evaluate the difference in average age of vaccinated and non-vaccinated groups.

Chi-square test and Fisher's exact test were used to analyse the data and evaluate the correlation between sex and the number of seropositive individuals in pigs and dogs, and also to evaluate the correlation between being kept mainly outdoors or indoors and seroprevalence among dogs.

RESULTS

Control procedure - inactivation and ELISA

The trial heat inactivation resulted in the denaturation of all the samples tested. Out of the 14 samples collected from dogs in Can Tho and used in the control ELISA, 10 were previously tested negative and four were tested positive for JEV according to the HI testing performed in Vietnam. Despite attempts to mechanically separate the samples and soaking the samples in PBST overnight, none of the trial samples were positive when tested with the competitive IgG ELISA described above. The positive control that was inactivated and tested showed an inhibition similar to the negative controls, the rate of inhibition being lower than 20%.

HI results

Pigs

The age of the pigs varied between 6 and 7.5 months, with an average age of 6.6 months and a median age of 6.5 months. Fifty two per cent (n=92) of the samples were taken from male pigs. All the animals were hybrids between different breeds.

Table 1. Results of HI analysis of pig samples divided by titre

Titres	No of samples	(%) of positive samples
1:20	7	4.4
1:40	7	4.4
1:80	17	10.7
1:160	27	17.0
1:320	24	15.1
1:640	25	15.7
1:1280	20	12.6
1:2560	24	15.1
1:5120	8	5.0
Total	**159**	**100**

In total, 90.3% (n=159) of the tested pigs originating from Can Tho were positive for JEV with titres ranging from 1:20 to 1:5120, with a median titre of 1:320. The distribution of the samples by titre is shown in Table 1. The proportion positive samples differed only slightly between districts, ranging from 86.4% to 92.9%. The distribution of samples and positive pigs, based on districts, are shown in Figure 5 and Table 2. Ninety one point seven per cent (n=77) of the female pigs and 89.1% (n=82) of the male pigs were JEV positive (Table 3). This difference in seroprevalence between the sexes was not found to be statistically significant.

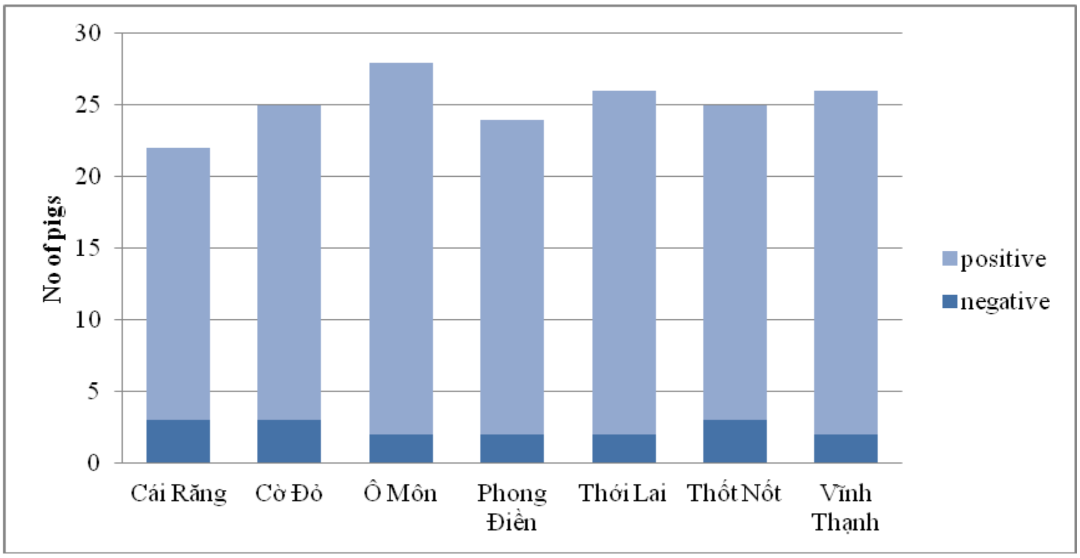

Figure 5. Results of HI analysis of pig samples divided by district.

Table 2. Results of HI analysis of pig samples divided by district

District	Negative	Positive	Total no of pigs	(%) positive
Cái Răng	3	19	22	86.4
Cờ Đỏ	3	22	25	88.0
Ô Môn	2	26	28	92.9
Phong Điền	2	22	24	91.7
Thới Lai	2	24	26	92.3
Thốt Nốt	3	22	25	88.0
Vĩnh Thạnh	2	24	26	92.3
Total	**17**	**159**	**176**	**90.3**

Table 3. Results of HI analysis of pig samples based on sex

Sex	Negative	Positive	Total no of pigs	(%) positive
Female	7	77	84	91.7
Male	10	82	92	89.1
Total	**17**	**159**	**176**	**90.3**

Dogs

The age of the dogs varied between 3 and 216 months. The average age was 31 months and the median age was 20 months. The sex distribution was 56.7% (n=72) female and 43.3% (n=55) male dogs. A majority of the dogs (89.8%, n=114) were mainly kept indoors and 10.2% (n=13) were mainly kept outdoors.

Table 4. Results of HI analysis of dog samples collected from dogs originating from Can Tho city province divided by district

District	Negative	Positive	Total no of dogs	(%) positive
Cái Răng	23	5	28	17.9
Ninh Kiều	75	22	97	22.7
Ô Môn	1	0	1	0.00
Thốt Nốt	1	0	1	0.00
Total	**100**	**27**	**127**	**20.3**

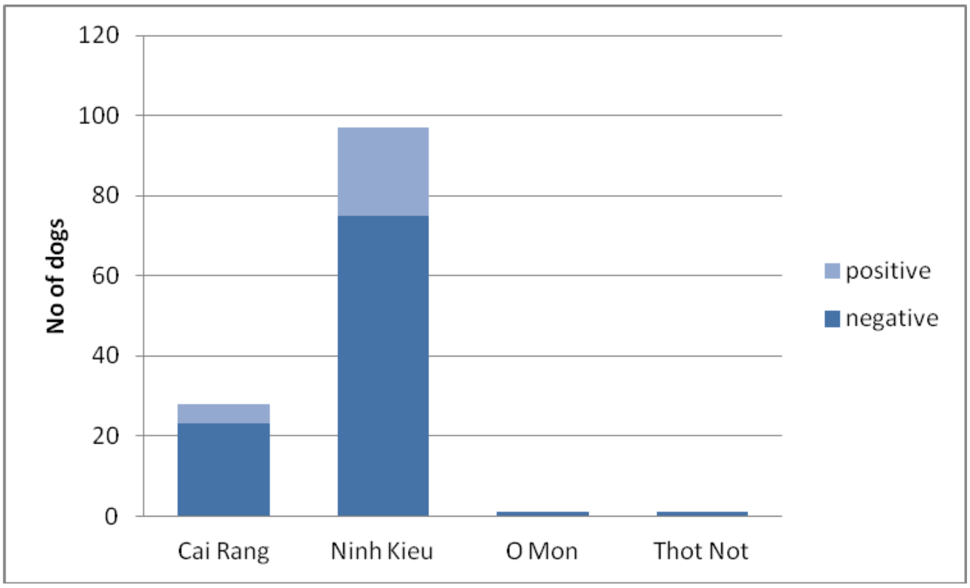

Figure 6. Results of HI analysis of dog samples collected from dogs originating from Can Tho city province divided by district.

A total of 20.3% (n=27) of the samples from Can Tho were JEV positive with titres ranging from 1:20 to 1:320, with a median titre of 1:40. The distribution of the titres is shown in Table 5. The distribution of samples and positive dogs based on districts are shown in table Figure 6 and Table 4. There was a significantly (p=0.01) higher percentage of positive dogs in the group kept mainly outdoors (61.5% positive, n=8) than in the group kept indoors (16.7% positive, n=19) (Figure 7). No significant differences were found when comparing the groups of male and female dogs or when comparing different age groups.

Table 5. Results of HI analysis of dog samples collected from dogs originating from Can Tho city province divided by titre

Titres	No of samples	(%) of positive samples
1:20	12	44.4
1:40	6	22.2
1:80	7	25.9
1:160	1	3.7
1:320	1	3.7
Total	**27**	**100**

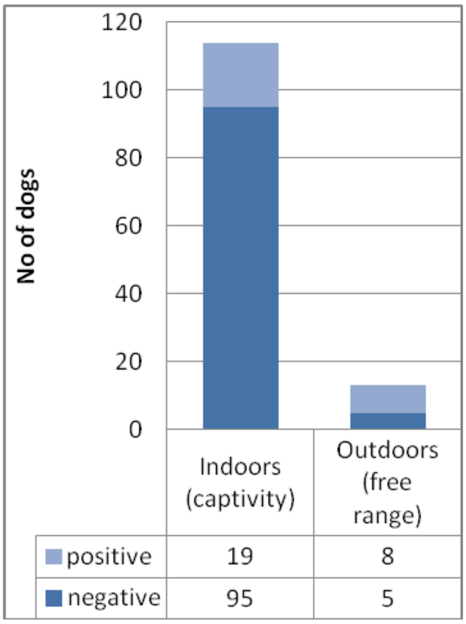

Figure 7. Results of HI analysis of dog samples collected from dogs originating from Can Tho city province showing the distribution of positive and negative dogs mainly being kept indoors or mainly being kept outdoors.

Questionnaires

A total of 82 people answered the questionnaire, 24 tourists or expatriates (referred to as the tourist group) and 58 Vietnamese (referred to as the Vietnamese group). The total age span of both groups was 14-63 years of age. Gender distribution for both groups were 43.3% (n=38) females and 56.7% (n=44) males. Approximately 68.3% (n=56) had heard of JE and 34.1% (n=28) were vaccinated against JE. People under the age of 30 were significantly ($p<0.0001$) more likely to respond that they were vaccinated against JEV than the older age groups as shown in Table 6. In the age group <30 years 59.5% stated that they were vaccinated. The mean age in the group stating that they were vaccinated against JE (n=27) was significantly ($p<0.0001$) lower (mean age of 28.0 years) than in the non-vaccinated group (n=49, mean age of 39.4 years).

Table 6. Vaccination status of different age groups, (p<0.0001)

	<30 years	**>30 years**	**≥50 years**	**Total**
Vaccinated (% of age group)	22 (59.46)	5 (20.00)	1 (6.67)	28
Not vaccinated (% of age group)	15 (40.54)	20 (80.00)	14 (93.33)	49
Total (% of age group)	**37 (100)**	**25 (100)**	**15 (100)**	**77**

Eighty six point six per cent (n=71) used one or more precautions to avoid being bitten mosquitoes, the most common precautionary method taken being mosquito repellent used by 63.4% (n=52) followed by mosquito net (30.5%, n=25), long clothing (14.6%, n=12) and other (13.4%, n=11). Figure 8 shows the precautionary measures taken based on group. When comparing whether people who had heard of JE used different precautions to a higher degree than those who had not heard of JE, it was found to be statistically significant that people who had heard of JE used mosquito repellent to a higher extent (70.9%, n=39) than people who had not heard of JE (48%, n=12) (p=0.02). There were no other significant differences in relation to other precautionary methods taken.

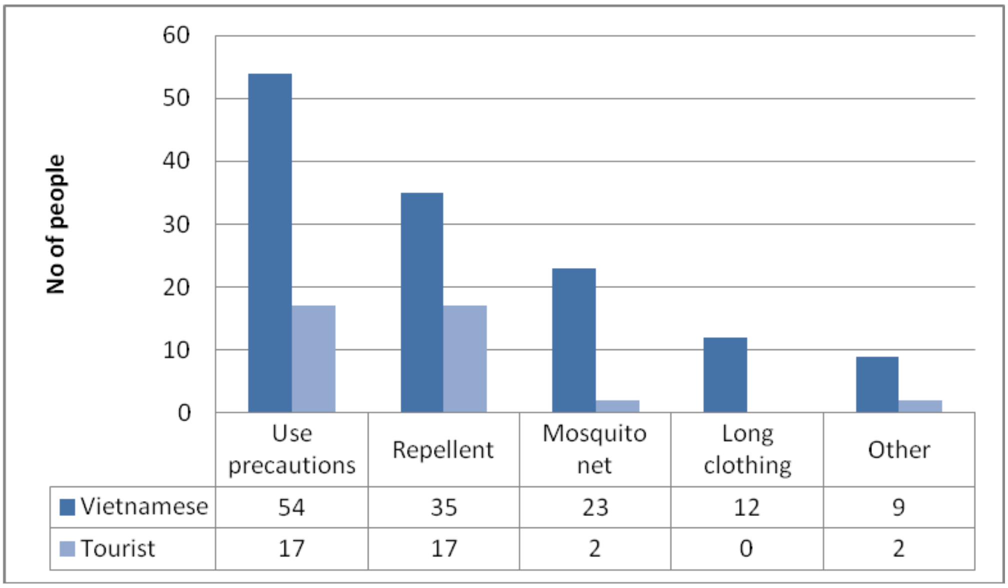

Figure 8. Precautionary methods taken to avoid being bitten by mosquitoes based on group.

In the tourist group, a majority (95.7%, n=22) responded that they were in Vietnam on vacation, while one (4.3%) person answered that he worked in Vietnam. The duration of their stay in Vietnam varied between 12 days and 12 years. Only two people stated that they were staying for a longer period than 30 days.

In the Vietnamese group, 57.4% (n=31) stated that they owned dogs. One (1.7%) person stated that he owned pigs. Thirty nine point seven per cent (n=23) stated that they lived close to stale water and 11.1% (n=6) lived close to rice fields.

When comparing the two groups it was found that the percentage of people who had heard of JE was significantly higher (p<0.0001) in the Vietnamese group, where 79.3% (n=46) stated that they had heard of JE, than in the tourist group, where only 41.7% (n=10) stated this.

There was also a significantly (p<0.002) higher percentage of people in the Vietnamese group (n=26, 44.8%) who responded that they were vaccinated against JEV than in the tourist group (n=2, 8.3%) (Figure 9).

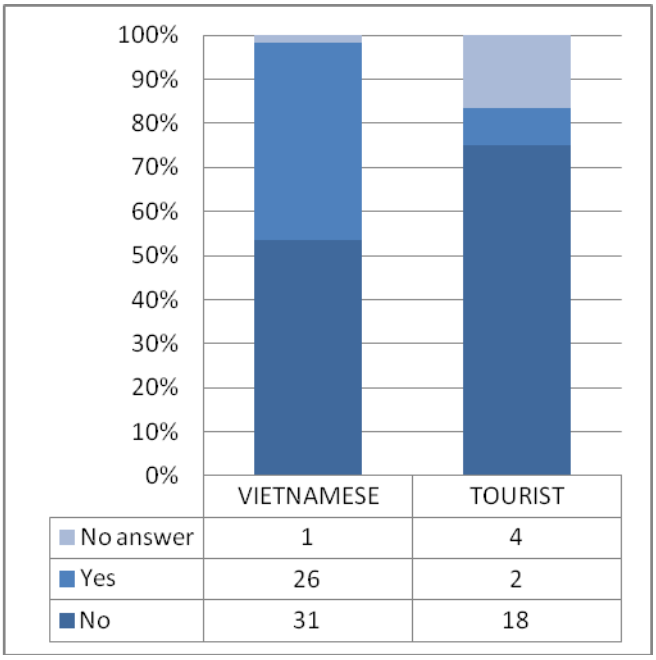

Figure 9. Percentage of people who were vaccinated against JE based on group (p<0.002).

DISCUSSION

General

In this study, sera from pigs and dogs in Vietnam were collected and analysed for the presence of JEV antibodies. The purpose was to examine the seroprevalence of JEV in these species within the urban parts of Can Tho city and Ho Chi Minh City, and also to evaluate if dogs could be used as sentinels for JEV and for assessing the risk that JEV may pose to people living in urban areas located in regions where JEV is endemic. We also wanted to look at how some risk factors may affect the risk that JEV poses to humans in these areas. The risk factors we wanted to look at was living close to pigs, because of their potential to act as reservoirs, and living close to rice fields or stagnant water, because these are the primary breeding grounds for some of the mosquitoes spreading JEV (Reuben 1971, Sunish and Reuben 2001). Other reasons for looking at closeness to rice fields and stagnant water as risk factors was because previous research has shown that vectors needed for transmitting JEV are present in Can Tho, that some of the vectors do carry the virus (Thu *et al.* 2006, Lindahl et al. 2012b), and because the presence of rice fields within the urban area as well as the annual flooding may be contributing to the presence of JEV vectors in Can Tho (Epstein 2001, Correia *et al.* 2013).

The results of the HI analysis showed a seroprevalence of more than 20% in dogs and more than 90% in pigs. The high seroprevalence of JEV in pigs in this study confirms the previous research from the same region indicating a JEV seroprevalence of 60-100% in pigs originating from Can Tho city province (Lindahl *et al.* 2012a, Thu *et al.* 2006b, Lindahl 2012). Using an IgG ELISA, Lindahl (2012) showed that almost 100% of pigs kept in the urban areas of the Ninh Kieu district were positive for JEV.

Pigs have traditionally been used for JEV surveillance in epidemic areas (Rosen 1986). Pigs seroconvert approximately 2-3 weeks before humans, when JEV is introduced into a new area (Daniels *et al.* 2002). They may however not be an ideal candidate for surveillance in urban environments located in endemic areas for several reasons. First, pigs have the potential to function as a reservoir for JEV, thus posing a risk to humans in themselves. Furthermore pigs are usually kept somewhat separated from the human habitat, and partly attract different mosquito species than the ones usually feeding off humans. Thus, pigs may not accurately reflect the risk JEV poses to humans (Moore *et al.* 1993). The results of this study supports previous research which has found that more than 90% of the pigs older than 6 months kept in endemic areas often are seropositive to JEV (Duong *et al.* 2011, Lindahl 2012). A high rate of seroconversion occurring at a young age (majority of pigs included here were 6-7 months old) may pose difficulties to evaluate the risk factors behind infection. Because of the high infection rate in pigs it may also be hard to use them to detect changes in the infection pressure and therefore their use to evaluate the risk JEV poses to humans living in urban areas may be limited.

In urban environments located in endemic areas dogs may be better sentinels and may be valuable in evaluating the risk that JEV poses to humans. There are several factors making the dog a good sentinel for JEV. Dogs fulfil the main criteria of being a good sentinel postulated by Moore *et al.* (1993) i.e. live close to humans, do not get sick from the infection, do not develop a viraemia high enough to pass the virus to a new vector, but do develop an antibody titre which is easily measurable during a long period of time, as shown by Shimoda *et al.* (2011). Furthermore it has been shown that *Cx. quinquefasciatus*, the vector most likely to pose a threat to humans due to its anthropophilic feeding pattern, also feed off dogs to a high extent (Mitchell *et al.* 1973, Reuben *et al.* 1992, Garcia-Rejon *et al.* 2010, Alencar *et al.* 2012). In this study it was found that a significant number of dogs (20.3%) were positive for JEV antibodies when tested using HI, thus indicating that dogs do seroconvert to a fairly high extent as a result of infection. This, together with the research done in Japan and Thailand by Shimoda *et al.* (2010, 2013), indicates that dogs do make good sentinels for JEV (Shimoda *et al.* 2010, 2011, 2013). In the research by Shimoda *et al.*(2010) serological analyses of 652 blood samples collected from dogs in different regions of Japan during 2006-2007 showed that 25% of the dogs had antibodies against JEV. In that study there was a large variation in the number of positive dogs found in different districts varying from 0% in some of the northern districts up to 61% in one of the southern districts. There were a significantly higher percentage of dogs infected with JEV living in the rural areas (43%) than in the urban areas (21%) in Japan. Dogs kept strictly outdoors showed a higher seroprevalence for JEV (45%), than dogs kept strictly indoors (8%). In the northern regions of Japan where the prevalence of infected pigs was lower, fewer dogs had antibodies than in the southern part where the prevalence of infected pigs was higher. When using an IgG-ELISA and researching seroprevalence of JEV in dogs in Bangkok, Thailand, Shimoda *et al.* (2013) showed that approximately 66% of the dogs kept outdoors were seropositive, while none of the dogs kept indoors tested positive for JEV antibodies.

The HI results in our research also showed a significantly higher percentage of positive samples taken from dogs mainly kept outdoors when compared to dogs mainly kept indoors (p=0.01). This is reasonable because dogs mainly kept outdoors will probably be subjected to virus-carrying vectors to a higher extent than dogs mainly kept indoors. The fact that 16.7% of the dogs mainly kept indoors were positive to JEV in this study, is strongly indicative of JEV posing a risk to humans living there, especially young children who are not vaccinated against JE. Dogs mainly being kept indoors may have a living pattern similar to that of their owners, i.e. spend almost the same amount of time, or more, as their owners indoors, and thus be subjected to the same ratio of outdoor and indoor vectors as their owners. Because of this they may fairly well reflect the risk that virus-carrying vectors may pose to their human owners. It is important to keep in mind that, although dogs do attract some of the same vectors as humans to a high extent (Mitchell *et al.* 1973, Reuben *et al.* 1992, Garcia-Rejon *et al.* 2010, Alencar *et al.* 2012), the measures taken by humans to avoid mosquito bites, as well as the fact that dogs may be more tolerant to mosquito bites than humans, may cause dogs to be more exposed. This could bias the use of dogs to be used for evaluating the risk JEV poses to humans, and more research is needed to determine to what degree the risk for dogs and humans getting infected with JEV correlate.

Since our results show that the seroprevalence (20.3%) was only moderately high in dogs they may, in contrast to pigs, also be suitable to use to detect changes in infection pressure.

In the present study, antibody titres in the positive dogs ranged from 1:20 to 1:320 with a majority (66.6%, n=18) having a titre of either 1:20 or 1:40. Only 7.4% of the dogs had titres of 1:160 or higher. The positive pigs had antibody titres ranging from 1:20 to 1:5120, with a median of 1:320. It is well known that flaviviruses have a tendency to cross-react with other flaviviruses. This is especially true if the viruses belong to the same serological group (sero-group), such as WNV and JEV. However, it has been shown that dengue virus, despite belonging to another sero-group, has a tendency to cross-react with JEV (Madrid and Porterfield 1974, Kimura-Kuroda and Yasui 1986, Calisher *et al.* 1989, Kuno 2003, Yamada *et al.* 2003). The high IgG antibody titres caused by dengue virus infection might be the reason for its cross-reaction with JEV (Innis *et al.* 1989, Unni *et al.* 2011). Because HI measures both IgG and IgM antibodies, the risk for cross-reactions between different flaviviruses cannot be disregarded in the interpretation of the HI results. Due to this, especially the dog samples showing low titres of JEV antibodies should be interpreted with some caution, and although the HI results do indicate that JEV does pose a risk to humans living in Can Tho city urban area, further studies are needed.

An attempt was made to perform a competitive IgG ELISA on serum after high-temperature inactivation. Since the heat inactivation resulted in denaturation of all samples, and no free fluid was possible to separate from the coagula, the samples were not possible to use for an ELISA. It is well known that serum albumin starts coagulating around 60ºC. Although at this temperature the coagulation is only partial, increasing temperatures result in further denaturation (Schäfer 1882, Gellis *et al.* 1948). In a study by Chick (1910) on egg albumin, it was shown that for every 1ºC rise in temperature the heat coagulation velocity increases by 1.91 times, resulting in the rate increasing 635 times for a 10ºC rise in temperature. It was also shown that time was a factor, with increased coagulation occurring proportionally to the increase in time. According to OIE, a temperature above 56ºC for 30 minutes is sufficient to destroy JEV, and the thermal inactivation point for JEV is 40ºC (OIE 2013). Based on this, a lower temperature and shorter time of inactivation would be recommended if the samples are to be used for an ELISA.

Although we did not succeed in getting any ELISA results in this research, Shimoda *et al.* (2013) have shown that the indirect IgG ELISA they used to test experimentally infected as well as naturally infected dogs was, although not very sensitive (82%), was highly specific (98%) when compared with virus neutralization test. ELISA testing does not demand handling of viable virus, requires only a small amount of sera (compared to HI), can be performed on a high number of samples, and is fairly easy to perform, thus giving it advantages to virus neutralization test and HI test. More testing of dogs in urban studies using an ELISA is recommended to further evaluate the value of using dogs for surveillance of JEV in urban areas.

At the Saigon Pet Clinic 65 blood samples from dogs were collected. These were not available to the researchers at Can Tho University and were because of this not included in the HI testing. Since all the samples sent to Sweden coagulated in the heat inactivation and an ELISA could not be performed, the JEV status of the dogs originating from Ho Chi Minh City is unknown. The data collected about these dogs was therefore excluded in this thesis.

Questionnaire and data collection

In the survey, there was a significantly ($p<0.0001$) higher percentage of people in the Vietnamese group who stated that they had heard of JE than in the tourist group. This difference can probably be partially explained by the fact that JE is a disease that is endemic to the part of Vietnam where the questionnaires were handed out. But it cannot be disregarded that a high proportion of the Vietnamese questionnaires were answered by students who were well educated and might have had more opportunities to hear of the disease, than a person chosen at random. There was also a significant ($p<0.002$) difference between the groups in the number of people who stated that they were vaccinated against JE, with 44.8% of the

Vietnamese group stating that they were vaccinated against JE compared to only 8.3% in the tourist group stating this. In 1997 a national vaccination program started in Vietnam in which school children in the most high risk areas were immunized against JE. By 2007 the program included Vietnamese children in 65% of the districts (Yen *et al.* 2010). Vaccination for tourists is usually recommended only if you plan to stay in an endemic area for more than 30 days, are going to an area where there is a current outbreak, or if you are visiting an epidemic area (Tiroumourougane *et al.* 2002, Dutta *et al.* 2010). It is explicitly not recommended for travellers who plan to stay only a short time in urban areas, even if these are located in an endemic region (CDC 2012). Since only two tourists stated that they were staying in Vietnam for longer than 30 days it is thus not surprising that there were a significantly higher percentage of vaccinated people in the Vietnamese group than in the tourist group. The national vaccination program may also explain why there was a significantly ($p<0.0001$) higher percentage of vaccinated people in the youngest age group (<30 years) than in the older ones.

Although more research is needed to better evaluate the risk that JEV poses to humans in Can Tho city urban area and other urban areas in regions where JEV is endemic, the results of our research indicates that JEV does pose a risk to humans in these areas. And because it is clearly advised against vaccination when visiting urban areas a revision of the vaccination recommendations for travellers visiting endemic urban areas may therefore be recommended in the future.

The survey conducted only included a small number of people (n=82), and people belonging to the Vietnamese group were not selected through a correct random selection, but rather based on the presence of an interpreter who could ask them to fill out the questionnaire. Some of the questions regarding having pigs, rice fields, or stagnant water in the vicinity of the household may not always be known to the person answering the questionnaire. Furthermore these questions could have been more clearly defined and could also have included the alternative answer “I don’t know” in addition to “Yes” and “No”.

Several more factors may have influenced this research and should also be taken into account. When doing research in a country where an interpreter is needed to it is important to keep in mind that translations may alter the meaning of a word. This may result in the same question being interpreted in different ways or even being translated incorrectly, thus resulting in someone answering the “wrong” question due to a misunderstanding. For example, in this research an exclusion criterion, stating that dogs should not be younger than 6 months, was used. But due to either misunderstanding, misinterpretation, or maybe due to a lack of communication three dogs under the age of 6 months were sampled.

Regarding the data collection, some discrepancies were found when comparing the collected data acquired on site to the data later received in connection to the HI results. The discrepancies found were regarding for example the ages of pigs and addresses of dogs. Questions regarding the risk factors (dogs kept in households close to pigs or living close to stagnant water or rice fields) were not clearly defined thus giving considerable room for individual interpretation. An alternative approach would have been to have defined close to as within a specified radius of for example 100 meters. It could also have aided to have a clear definition of what "kept outdoors" and "kept indoors" meant, i.e. specifying how many hours per day of time a dog would have to be kept indoors to be classified as belonging to the indoor group. This latter data was included in the statistical analysis, but instead of using the terms "kept indoors" and "kept outdoors", the terms "mainly kept indoors" and "mainly kept outdoors" were used, to indicate that definitions were used in a wider sense. Furthermore it might have been valuable to have collected information about the dogs' health status in the questionnaire. Manifest disease was not an exclusion criterion and samples were collected from dogs coming in for their yearly vaccination as well as from severely ill dogs. Although no information would have been available of the disease-causing agent, it might have been valuable to analyse whether dogs showing signs of illness were JEV positive to a higher extent than those not showing signs of disease.

Because of the discrepancies in data some data was excluded from the statistical analysis or from this thesis, for example no comparison was made comparing the ages of pigs and seroprevalence. Partly because of the discrepancies in the data concerning the addresses of dogs, and the poorly defined questions regarding risk factors in the vicinity of the habitat of the dogs, and partly because of the fact that people might not be aware of if they live close to these risk factors, this was not analyzed further Thus no evaluation of risk factors was possible.

In regards to some of the data ascertained from official sources it can be challenging to acquire accurate and up-to date statistics on animal densities within city areas. It was not possible to find any statistics of the number of dogs living in the city, but due to a new legislation demanding that all dogs and cats have to be registered, this data should be possible to acquire in the future.

The official reported pig density in Ninh Kieu 2011 was 64 pigs/km^2, but the statistics only included pigs registered in two of the wards, indicating that pigs were not kept in the other wards of the Ninh Kieu district, whereas pig keeping households could be found in other wards as well during this study as in previous studies by Lindahl et al. (2012). The reasons for this may be that official statistical data are sometimes lagging or because of people not reporting to the authorities when they acquire pigs.

CONCLUSIONS

In this study it was possible to show that both dogs kept indoors and outdoors in the urban area of Can Tho city were seropositive for JEV. This further indicates the risks for urban transmission to humans, and emphasises the needs of more studies in the area.

We found that dogs seroconvert to a moderately high extent and, since they also meet the requirements postulated by Moore *et al.* (1993), could be considered suitable sentinels for

JEV, and likely can be used to evaluate the risk that Japanese encephalitis poses to humans in urban areas. Dogs may also be suitable to detect changes in infection pressure.

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