Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

**Faculty of Veterinary Medicine
and Animal Science**

Japanese encephalitis in small-scale pig raising in rural Cambodia

Seroprevalence, reproductive disorders and disease awareness

Ellinor Henriksson

Uppsala

2020

Degree Project 30 credits within the Veterinary Medicine Programme

Japanese encephalitis in small-scale pig raising in rural Cambodia

Seroprevalence, reproductive disorders and disease awareness

Ellinor Henriksson

***Supervisor**: Gunilla Ström Hallenberg, Department of Clinical Sciences*

***Assistant Supervisor**: Johanna Lindahl, Department of Clinical Sciences*

***Examiner**: Ulf Magnusson, Department of Clinical Sciences*

Degree Project in Veterinary Medicine

***Credits**: 30 HEC*
***Level**: Second cycle, A2E*
***Course code**: EX0869*
***Course coordinating department:** Department of Clinical Sciences*

***Place of publication**: Uppsala*
***Year of publication**: 2020*
***Online publication**: https://stud.epsilon.slu.se*
***Cover illustration:** Photo by Rebecca Söderberg*

***Key words**: Japanese encephalitis, JE, Japanese encephalitis virus, JEV, Cambodia, pig, swine, seroprevalence, reproduction, disease knowledge, mosquito-borne diseases, prevention, vaccination*

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Veterinary Medicine and Animal Science
Department of Clinical Sciences

SUMMARY

Japanese encephalitis (JE) is endemic in several Asian countries, including Cambodia. It is a neurologic disease caused by the mosquito-borne virus JEV, which infects a wide range of vertebrate hosts. In humans, infection is usually subclinical, but one out of around 250 infected people develop acute encephalitis. Around 20–30% of the severe clinical cases are fatal, and up to half of the survivors are faced with permanent neuropsychiatric sequelae. Japanese encephalitis is mainly considered a childhood disease of rural areas. Pigs serve as amplifying host and develop high-level viremias following infection. They rarely develop clinical disease, although infection in adult pigs can cause reproductive disorders, such as abortions, stillbirths and infertility. Thus, JEV could have a major impact on the profitability of pig rearing.

Cambodia is a low-income, Southeast Asian country with 16.2 million people and 1.76 million pigs (2018 estimates). Pork is the most consumed type of meat, and many households keep pigs. Most pig producers are smallholders that live in rural areas, where JEV is likely to circulate. The pigs are usually kept near human dwellings, which increases the risk of infection in humans. In 2007, the incidence of clinical JE in Cambodia was 11.1 per 100,000 children under 15 years of age. Sub-national vaccination of children was initiated in 2009, and a national vaccination programme was established in 2016. The infection rate and seroprevalence in pigs has been estimated to be high in both rural and peri-urban areas in Cambodia. However, JEV circulation among pigs has only been studied in the southern part of the country.

In this study, 139 pig smallholders were visited in rural parts of Kampong Thom, Preah Vihear, Ratanakiri and Stung Treng provinces, north-eastern Cambodia. They were interviewed about pig management, occurrence of reproductive disorders, knowledge of JE and mosquito-borne diseases in general, and use of JE preventive measures, such as mosquito protection and vaccination. Pigs over three months of age (to avoid interference of maternal antibodies) were sampled for blood, and sera were analysed for JEV antibodies. Information about sex, age, breed and history of reproductive disorders was recorded for each sampled pig. In total, 242 pigs were sampled.

The apparent seroprevalence was 89.1% in pigs between three and six months of age and 100% in pigs over six months of age. In total, 93.0% of the pigs were positive. There were no significant associations ($p<0.05$) between serologic status and breed, housing system, proximity to rice fields, reproductive disorders, or protection of pigs from mosquitos. Province appeared to be associated with seroprevalence ($p<0.001$), but very few test results were valid for Preah Vihear, where the prevalence was lowest. All families used mosquito protection, almost all respondents knew that mosquitos can transmit diseases, and over two-thirds had heard of JE. The JE vaccination coverage in children appeared to be high in all provinces.

The results of this study suggest that JEV transmission is intense, not only in southern Cambodia, but in north-eastern, rural areas as well. Fortunately, people are well-aware of mosquito-borne diseases and protect themselves from mosquito bites, and many children are vaccinated against JE. Nonetheless, it is important that national vaccination is continued.

CONTENT

INTRODUCTION

Japanese encephalitis (JE) is a serious neurologic disease and a major health concern in many Asian countries, including Cambodia (WHO, 2019). It is caused by JEV, a mosquito-borne flavivirus that infects several vertebrate species, although mainly pigs, horses and humans develop clinical symptoms (van den Hurk *et al*., 2009). The major mosquito vectors prefer to breed in rice fields, and pigs are considered important amplifying hosts. Infection in humans may cause encephalitis and death, or neuropsychiatric sequela among survivors (Solomon *et al*., 2000). The disease is generally considered rural, and proximity to rice fields and pigs are associated with increased risk of transmission (van den Hurk *et al*., 2009).

In 2018, Cambodia had a population of 16.2 million people, of which 12.4 million resided in rural areas (FAO, 2019a). Rice cultivation and pig raising are common practices, especially in the countryside (Samkol *et al*., 2008). Many families are smallholders that keep their pigs close to their homes. Therefore, a major part of the population is in risk of infection. Pigs usually do not show any overt signs of disease, but infection in sows and boars may cause reproductive disorders such as abortions, stillbirths and infertility (Williams *et al*., 2019). Thus, JEV could have a negative impact on the livelihoods of farmers, as pig raising generates both income and food.

The purpose of this study was to determine the seroprevalence of JEV in smallholder pigs in rural parts of Kampong Thom, Preah Vihear, Ratanakiri and Stung Treng provinces, Cambodia. Although JE is considered endemic (Touch *et al*., 2009) and most pigs seroconvert before six months of age (Duong *et al*., 2011), the circulation of JEV among pigs has not been studied in the northern part of the country before. Another aim of the study was to identify possible associations between serologic status and animal characteristics, pig management and reproductive disorders. Lastly, smallholders were asked questions about JE, mosquito-borne diseases in general and preventive measures to find out how well-informed people are and if they protect themselves from mosquito bites or through JE vaccination.

LITERATURE REVIEW

Country facts about Cambodia

Cambodia is a Southeast Asian country bordering to Thailand in the northwest, Laos in the north and Vietnam in the east. The climate is tropical with monsoon rains but little seasonal temperature variation; the rainy season begins in May and ends in November, and the dry season begins in December and ends in April (CIA, 2020). Low, flat plains dominate the landscape, but mountains are found in the southwest and north. There are almost 3 million ha of rice paddy fields (FAO, 2020a), covering 17% of the land area (CIA, 2020). In 2017, the agriculture, forestry and fishing sector generated almost 5.2 billion USD, which made up 23.4% of the gross domestic product that year (FAO, 2019b). Other important sectors are garment exports and tourism, which have driven Cambodia's economy forward the past two decades; an average growth rate of eight percent between 1998 and 2018 has made Cambodia one of the fastest-growing economies in the world (The World Bank, 2019). In 2015, Cambodia reached lower middle-income status. Still, it remains one of the poorest countries in Asia. Although the poverty rate has fallen from 47.8% in 2007 to 13.5% in 2014, around 4.5 million people remain near-poor and risk falling back into poverty. The vast majority of the poor live in the countryside.

Japanese encephalitis, an important zoonosis

Epidemiology

Despite implementation of childhood vaccination programmes in many countries, Japanese encephalitis virus (JEV) remains the most important cause of human viral encephalitis in Asia (Campbell *et al*., 2011; WHO, 2019). Japanese encephalitis was first reported in Japan in 1924 during one of several epidemics of encephalitis described from the 1870s onwards (Erlanger *et al*., 2009). It was then reported on the Korean Peninsula in 1933, the Chinese Mainland in 1940, and in several other countries with Pakistan as the furthest extension in the west, northern Australia in the south, and south-eastern Russia in the north. Today, 24 Asian and Western Pacific countries, with a total population of over 3 billion people, are considered endemic, including Cambodia and its neighbouring countries (Campbell *et al.*, 2011). Approximately 67,900 clinical cases occur annually (incidence 1.8 per 100,000), an estimate based on studies on JE incidence in these countries published between 1985 and 2010. The true incidence is difficult to estimate since available data are sparse and partially derived from inadequate hospital-based surveillance. Only around one tenth of the cases are believed to be reported to the World Health Organization (WHO), which may be explained by varying quality of surveillance and availability of diagnostic laboratory testing. The majority of cases (75%) occur in children under 15 years of age (incidence 5.4 per 100,000). In Cambodia, 583 children in this age group were estimated to suffer from clinical JE infection in 2007 (incidence 11.1 per 100,000), however, the true incidence is probably underestimated (Touch *et al*., 2009; Tarantola *et al.*, 2014). Among the annual cases of JE, approximately 13,600 to 20,400 cases are fatal, and 14,300 to 27,200 of the survivors develop long-term neuropsychiatric sequelae (Campbell *et al.*, 2011). In 2005, the global burden of JE was estimated to be approximately 266,000 to 1,859,000 disability-adjusted life years (DALYs), a summary measure of premature mortality (years of life lost) and disability (years lived with disability, weighted by severity) (LaBeaud *et al.,* 2011).

Japanese encephalitis is strongly associated with irrigated rice agriculture and pig farming (Keiser *et al.*, 2005; Liu *et al.*, 2010). Cases mainly occur in rural areas (van den Hurk *et al.*, 2009), although there is evidence of virus circulation in peri-urban and urban areas as well (Lindahl *et al.*, 2013; Cappelle *et al.*, 2016; Di Francesco *et al.*, 2018). There are two major seasonal patterns of JE; in northern areas with temperate and subtropical climate, the disease occurs in outbreaks, mainly during the summer season (epidemic pattern), whereas in southern areas with tropical climate, sporadic cases occur throughout the year, but tend to peak during the rainy season (endemic pattern) (van den Hurk *et al.*, 2009). Japanese encephalitis is generally considered a childhood disease and is found more often in males than females (Umenai *et al.*, 1985). In epidemic areas, JE is mainly observed in children and young adults, but all age groups may be affected if outbreaks occur in new areas or after long periods with no virus activity (van den Hurk *et al.*, 2009). However, in temperate and subtropical countries with established childhood vaccination programmes, cases are most common in the elderly. In endemic areas without vaccination programmes, most adults have acquired active immunity through repeated exposure to JEV, and disease is mainly observed in infants and young children. A study conducted by Touch *et al.* (2009) confirmed JE as endemic in Cambodia. Sentinel surveillance was carried out between 2006 and 2008 at six different hospitals throughout Cambodia for children aged 15 years and younger presenting with meningo-encephalitis, and cases were found year-round and at all sentinel sites. Of all cases of meningoencephalitis, JEV was diagnosed as the causative agent in 19%. Children up to 12 years of age were dominantly affected (95% of cases), with the mean age being 6.2 years. A slight majority (56%) of cases occurred in males. The hospitals were located in six different provinces (Battambang, Kampong Cham, Phnom Penh, Siem Reap, Svay Rieng, and Takeo), and cases resided in all of them, as well as in eight other, adjacent provinces. Although cases occurred year-round, there was a moderate increase in cases mid-year, probably reflecting the number of vectors which increases during the rainy season.

Japanese encephalitis virus

Japanese encephalitis virus exists as a single serotype and belongs to the genus *Flavivirus* in the *Flaviviridae* family (Mackenzie *et al.*, 2006; Simmonds *et al.,* 2017). It is part of the JEV serological complex comprising 10 antigenically related, mosquito-borne flaviviruses: Alfuy, Cacipacoré, JE, Koutango, Kunjin, Murray Valley encephalitis, St. Louis encephalitis, Usutu, West Nile, and Yaoundé viruses, with Alfuy and Kunjin viruses being subtypes of Murray Valley encephalitis and West Nile viruses, respectively (Poidinger *et al*., 1996; Mackenzie *et al*., 2006; Simmonds et al., 2017). Japanese encephalitis virus, as well as the other members of the genus *Flavivirus*, has a positive-sense single-stranded RNA genome encoding three structural proteins (capsid [C], precursor membrane [PrM], and envelope [E]) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Simmonds *et al.*, 2017). There are five known genotypes of JEV based on the nucleotide sequence of the E gene: G1, GII, GIII, GIV, and GV (Solomon *et al.*, 2003). Genotype I is further divided into two clades: GI-a and GI-b (Schuh *et al.*, 2014). Genotypes IV and V represent the oldest, and most divergent, linages of JEV, whereas GI, GII and GIII evolved more recently (Solomon *et al.*, 2003). Japanese encephalitis virus probably emerged from an ancestral virus in the Indonesia-Malaysia region and subsequently spread across Asia. The prototype JEV strain, known as Nakayama, was isolated from the brain of a fatal human case in Japan in 1935 (Solomon *et al.*, 2000) and was recognized as GIII (Gao *et al*., 2019). This was the most commonly isolated genotype until the 1990s, when GI displaced it (Schuh *et al.*, 2014). Since then, GI has remained the dominant genotype throughout Asia (Schuh *et al.*, 2014; Gao *et al*., 2019). Genotypes I and III are the most prevalent and widely distributed genotypes, while GII, GIV and GV are mainly confined to tropical regions of Southeast Asia (Gao *et al*., 2019). In the geographical region comprising Cambodia and adjacent countries, GI, GII and GIII circulate (Solomon *et al*., 2003; Gao *et al*., 2019).

Transmission cycle

The primary vectors for transmission of JEV are mosquitos of the *Culex vishnui* subgroup, including *Cx. vishnui*, *Cx. pseudovishnui,* and *Cx. tritaeniorhynchus* (van den Hurk *et al*., 2009). The latter is considered most important as it is widely distributed over the geographic range of the virus (Rosen, 1986). Other species, such as *Cx. annulirostris, Cx. fuscocephala, Cx. gelidus*, *Cx. pipiens*, and *Cx. quinquefasciatus,* serve as secondary or regional vectors (Rosen, 1986; van den Hurk *et al*., 2009). In total, JEV has been isolated from over 30 mosquito species, including *Culex* mosquitos as well as species of other genera, such as *Aedes*, *Anopheles, Armigeres* and *Mansonia,* but not all are believed to transmit the virus to new hosts. The virus has also been isolated from midgets and ticks, although their importance in the transmission cycle of the virus is unknown (Mackenzie *et al*., 2006). The primary *Culex* vectors breed in rice fields, marches and other sources of standing water (Keiser *et al.*, 2005), bite during the night, particularly in the hours after dawn and before dusk (Amerasinghe & Indrajith, 1995), and are zoophilic, preferring to feed on animals rather than humans (Mackenzie *et al*., 2006; van den Hurk *et al*., 2009). Most JEV vectors are opportunistic and host availability influences feeding pattern; many prefer to feed on cattle rather than pigs, but in areas with large pig populations, porcine feeding rates may be high. Feeding pattern also seem to depend on previous exposure to a certain host. In a study carried out in northern Thailand, it was found that *Cx. tritaenio-rhynchus, Cx. pseudovishnui* and *Cx. gelidus* mosquitos preferred to feed on cattle rather than pigs after being exposed to cattle (Mwandawiro *et al.*, 2000). In the opposite scenario, only *Cx. vishnui* showed significant difference in feeding rates, preferring pigs to cattle. Feeding pattern was not inherited by the offspring of the mosquitos, but offspring generally chose to feed on cattle. The results suggest that host preference in JEV vectors may depend on behavioural conditioning, rather than genetic variability.

The mosquito vectors transmit the virus between several avian and mammalian vertebrate hosts. Wading birds of the family Ardeidae (herons and egrets) constitute the natural maintenance reservoir for JEV; they develop high levels of viremia without demonstrating clinical disease (Constable *et al*., 2017). Among domestic animals, pigs serve as important amplifying hosts; they are attractive for JEV vectors, develop a high-level and prolonged viremia capable of infecting mosquitos, and have a high seroprevalence rate (Mackenzie *et al*., 2006). Also, immune pigs are replaced by susceptible piglets at short intervals because of rapid turnover in pig populations. Infection with JEV can cause reproductive failure in adult pigs (abortions, mummified foetuses and stillbirths in sows, and infertility in boars) and neurological disease in piglets (Williams *et al*., 2019). In similarity to humans, horses risk developing fatal encephalitis following JEV infection (Constable *et al*., 2017). Despite their vulnerability to disease, humans and horses develop low levels of viremia and are considered dead-end hosts. Several other animals, including cattle, sheep, goats, dogs, cats, chickens, ducks, wild mammals, reptiles, and amphibians, acquire subclinical infection, and are unlikely to contribute to further viral transmission (OIE, 2019). However, the results from an experimental study on ducklings (*Anas platyrhynchos*) and chicks (*Gallus gallus*) suggest that young poultry may serve as amplifying hosts (Cleton *et al.*, 2014). Ducklings and chicks were subcutaneously inoculated with JEV inoculation at various ages from 2 to 42 days after hatching. Clinical signs of disease were observed in younger ducklings, but not in chicks, and levels of viremia were significantly higher in younger birds than older birds. In young birds, viremia levels were theoretically sufficiently high to infect JEV mosquito vectors.

A recent study showed that experimentally infected pigs can transmit JEV to other, susceptible pigs in a vector-free setting, and that pigs can become experimentally infected through oronasal inoculation with the virus (Ricklin *et al.*, 2016a). The study also found that infected pigs shed virus from the oronasal cavity, and that virus persist in the tonsils for a long time and in high levels after infection. To find out whether direct transmission between pigs may occur under field conditions, the hypothesis was tested through mathematical models using already collected serologic data from pigs in Cambodia (Diallo *et al.*, 2018). The results suggest that direct transmission may play a part in the natural transmission cycle of JEV in Cambodia, but they also confirm transmission by vectors as the primary mode of transmission. These findings may partly explain the overwintering mechanism of the virus observed in temperate areas, where the virus reappear each year after the cold season. Direct transmission may also contribute to the persistence of the virus in tropical areas during the dry season, when mosquito numbers are low. Earlier proposed explanations of overwintering include persistence and vertical transmission in vertebrates and mosquitos (van den Hurk *et al*., 2009). A third proposal, that does not suggest overwintering per se, is that the virus is reintroduced by migrating birds and bats and wind-borne mosquitos.

Human disease

Most people show no or only mild, flu-like symptoms following JEV infection, but approximately 1 in 250 develop severe clinical disease (WHO, 2015). After the bite of an infected mosquito, the virus amplifies peripherally in dermal tissue and lymph nodes, causing a transient viremia before crossing the blood-brain barrier into the central nervous system (Solomon *et al.*, 2000). The virus infects the neurons, causing neuronal cell death, either directly through viral multiplication within the cell or indirectly through massive inflammation (Ghosh & Basu, 2009). The incubation period is typically 5 to 15 days, and after a few days of non-specific febrile illness, which may include coryza, diarrhoea, and rigors, other symptoms follow, such as headache, vomiting, abnormal behaviour, reduced level of consciousness, seizures, and convulsions (Solomon *et al.*, 2000). Although the symptoms vary from case to case, the classic description of JE consists of the following features: a dull, flat, mask-like face with wide, unblinking eyes, tremor, generalised hypertonia, and cogwheel rigidity. In severe cases of encephalitis, the condition rapidly worsens with a decline in consciousness to coma (WHO, 2015). Later symptoms also include acute flaccid paralysis, either directly following the initial period of febrile illness, or in comatose patients (Solomon *et al.*, 2000). There is no specific antiviral treatment for JE, but supportive care is important as it relieves symptoms and stabilizes the patient (WHO, 2015).

Approximately 20–30% of the severe clinical cases are fatal, and 30–50% of survivors suffer long-term neuropsychiatric sequelae (Fischer *et al.*, 2008). In areas with better hospital facilities the case fatality rate is lower, but the number of patients with sequelae is higher (Solomon *et al.*, 2000). Fatal outcome and sequelae appear to be more common in children than adults (Schneider *et al.*, 1974). Deaths are mainly caused by aspiration, seizures, raised intracranial pressure, and hypoglycaemia (WHO, 2015). Sequelae include motor deficits, severe cognitive and language impairment, and recurrent convulsions (Solomon *et al.*, 2000). There are also sequelae that are more subtle, such as behavioural changes and learning difficulties. Hills *et al*. (2011) found that the majority of children surviving JE in Cambodia and Vietnam had sequelae of different severity. In approximately 10% of the survivors, sequelae were serious enough to be incompatible with independent living, such as daily seizures, incontinence, and assistance with toileting and dressing. In cases of moderate or mild sequelae, that are unlikely to make the child dependent, daily life is nonetheless affected; behavioural problems are common, as well as reduced school or work performance.

Infection in pigs

Within the first three days after infection with JEV, pigs develop a high-level viremia that lasts up to four days and is accompanied by fever and other generalized symptoms such as reduced appetite and manure production, and reluctancy to move (Scherer *et al.*, 1959; Ricklin *et al.*, 2016a; Ricklin *et al.*, 2016b). During the viraemic phase, the virus spreads rapidly through the body to the central nervous system and various other organs, including lymph nodes, spleen, ileum, bone marrow, thymus, kidneys and liver (Ricklin *et al.*, 2016a; Ricklin *et al.*, 2016b). In addition to the central nervous system, the virus displays strong tropism for secondary lymphoid tissue, especially the tonsils, where the viral loads remain high even after viremia ends, and virus persists for at least 25 days. The pigs shed virus through oronasal secretions between two to eight days post infection. At necropsy, histological lesions typical for viral meningoencephalomyelitis may be observed in the brain tissue. No changes are found in peripheral tissue, except for signs of immune activation in secondary lymphoid tissue.

Two to three days after the onset of viremia, the pigs start to produce immunoglobulin M (IgM) antibodies, reaching maximum levels about one week later, then declining to low levels within two to three weeks (Burke *et al.*, 1985). At a later stage, up to several weeks after infection, the production of immunoglobulin G (IgG) antibodies takes over (Scherer *et al.*, 1959). Once pigs have seroconverted, they remain immune up to three years (Geevarghese *et al.*, 1994). Pregnant sows and gilts that have immunity against JEV transfer maternal antibodies through the colostrum to their piglets, providing them with passive immunity (Scherer *et al.*, 1959). The maternal antibodies wane after time and are usually gone at two to three months of age in the pigs (Cappelle *et al.*, 2016), but may be present longer, in some cases until 7 months of age (Scherer *et al.*, 1959; Di Francesco *et al.*, 2018). Possible factors influencing the length of the passive immunity may be varying antibody titres and colostrum transfer in sows, age of the sow, litter size (affects the amount of colostrum consumed by each piglet), as well as individual variability of intestinal permeability and absorption of maternal antibodies in piglets (Di Francesco *et al.*, 2018).

Infection is generally subclinical in adult pigs but may cause reproductive disorders (Williams *et al.*, 2019). If pregnant sows or gilts become infected before 60 to 70 days of gestation, the virus can cross the placenta and infect the foetuses, causing abortions or resulting in litters with stillborn or mummified foetuses or live, weak piglets. Stillborn foetuses and live, weak piglets may exhibit the following lesions: hydranencephaly, widespread oedema and congestion of the brain, histological signs of encephalitis, subcutaneous oedema and congested lymph nodes (Desingu *et al*., 2016; OIE, 2019). In boars, the virus can infect the testicles, which become oedematous and congested, resulting in lowered count and motility of sperm (Williams *et al.*, 2019). Infertility is usually temporary, as most boars recover from these effects. There are few reports on encephalitis in postnatally infected piglets, but in an experimental study performed by Yamada *et al.* (2004), three-week-old piglets intravenously inoculated with JEV presented with fever, depression and tremors of the hindlimbs. At necropsy, their brains were swollen and oedematous, with histological signs of nonsuppurative encephalitis.

Risk factors for transmission

Since JEV is transmitted by mosquitos, vector abundance is an important factor associated with infection in both humans and pigs, along with other parameters influencing vector abundance. Therefore, the results of studies investigating relationships between transmission and risk factors are often confluent. In three studied areas in Sri Lanka, peaks of vector abundance were associated with concurrent or slightly lagged (one to two months) peaks of seroconversion in pigs, which was followed by an increase in reported cases of viral encephalitis in humans in two areas (Peiris *et al.*, 1993). Vector abundance was inversely correlated with altitude, as was seroprevalence in humans and domestic animals. When vector ecology was studied at three suburban sites in Bangkok, Thailand, the minimum infection rate in mosquito vectors (*Cx. tritaeniorhynchus* and *Cx. gelidus*) and seroprevalence in pigs were found to increase with vector abundance (Gingrich *et al.*, 1992). Rainfall correlated with all three parameters.

During a four-year period, mosquitos were collected in Kurnool district of Andhra Pradesh, India, along with meteorological data (Murty *et al.*, 2010). When analysing vector abundance and climatic variables, significant correlations were found between abundance of *Cx. tritaeniorhynchus* and *Cx. gelidus* and wind speed, maximum and minimum temperature, and rainfall. Vector abundance was inversely correlated with maximum and minimum relative humidity, although these results were not statistically significant. In Jieshou county, eastern China, the incidence of JE in the human population between 1980 and 1996 was studied in relation to climatic factors (Bi *et al.*, 2003). Monthly mean maximum and minimum temperature, together with monthly precipitation, had a significant relationship with the number of reported JE cases, with a one-month lag effect. Hsu *et al*. (2008) conducted a similar study in Taiwan and found that JE occurrence was significantly associated with temperature and rainfall in the previous one to two months, as well as pig density.

Type of habitat also influences vector abundance; the highest numbers of *Cx. vishnui* subgroup mosquitos are found in rice field areas, although they are present in all other habitats as well, ranging from forests to urban sites (Thongsripong *et al.*, 2013). Incidence of JE is strongly associated with irrigated rice agriculture, with more cases occurring in extensively irrigated areas and within families practicing rice cultivation (Keiser *et al.*, 2005). Lastly, vector abundance is associated with pig keeping, as observed by Lindahl *et al.* (2012a) when collecting mosquitos in urban households with and without pigs in Can Tho city, Vietnam. Vector abundance correlated with the presence of pigs, as well as pig density. Also, traps that were in closer proximity to pigs than humans contained higher numbers of mosquito vectors. In Bali, occurrence of JE in children was significantly associated with pig ownership by their family or next-door neighbours, and proximity (less than 100 m) of the household to a rice field (Liu *et al.*, 2010). To summarize, the results of several studies suggest that JEV transmission is associated with vector abundance, temperature, precipitation, elevation, irrigated rice agriculture, and pig farming.

Prevention and control

The lack of curative treatment, together with poor clinical outcome, makes vaccination an important preventive measure for JE (Fischer *et al.*, 2008). There are four main types of vaccines currently in use, all based on genotype III virus strains: inactivated mouse brain-derived vaccines, inactivated Vero cell-derived vaccines, live attenuated vaccines, and live recombinant (chimeric) vaccines (WHO, 2015). Because humans are dead end hosts, vaccination in the human population have no impact on the enzootic transmission cycle of the virus, and disease can still occur in susceptible individuals. High vaccination coverage is therefore necessary to eliminate human disease. In 2016, half of the JE endemic countries had established national or subnational vaccination programmes (Heffelfinger *et al.*, 2017), which have led to a significant decline in JE incidence (WHO, 2015). The WHO recommends all countries where JE is a recognized public health priority to implement national vaccination (WHO, 2015). It should also be considered in areas with suspected JEV transmission, such as presence of animal hosts, favourable ecological conditions, and neighbouring areas with known transmission. The best way to implement JE vaccination in endemic countries is to launch a one-time campaign targeting the population at risk (typically children under 15 years of age), followed by incorporation of the vaccine into the routine childhood immunization programme. In 2009, after a two-year sentinel surveillance study was made to outline the burden and epidemiology of JE in Cambodia, the government launched a subnational routine vaccination programme, targeting children aged 10 to 25 months in three provinces: Kampong Cham, Svay Rieng, and Takeo (PATH, 2010). In 2016, Cambodia had established a national routine vaccination programme with scheduled immunization starting at 9 months in children, using CD-JEV (Heffelfinger *et al.*, 2017), a live attenuated vaccine based on the SA 14-14-2 JEV strain (WHO, 2015).

Since pigs are amplifying hosts, and pig farming is suggested as a risk factor for vector abundance and JE in humans (Hsu *et al.*, 2008; Liu *et al.*, 2010; Lindahl *et al.*, 2012a), vaccination of pigs could reduce the risk of transmission to humans, in addition to preventing JEV induced reproductive disorders (Williams *et al.*, 2019). A compartmental model built to describe JEV transmission dynamics in north-western Bangladesh, where JE is endemic, suggests that vaccination of 50% of the pigs would result in an 82% reduction of annual JEV infections in pigs (Khan *et al.*, 2014). However, because of the high turn-over rate in pig populations, as pigs are generally slaughtered between six and eight months of age and new piglets are born continuously, vaccination needs to be repeated often and is economically challenging (Williams *et al.*, 2019). Also, the presence of maternal antibodies, which is difficult to predict since the duration of passive immunity varies in pigs, may interfere with vaccination, making it ineffective. Porcine vaccination with inactivated, mouse brain-derived, and live attenuated vaccines have been undertaken in Japan, Taiwan, Nepal, and Korea. The latter vaccine is more efficacious than the former against natural and experimental infection. Other possible preventive actions are to reduce contact between pigs and vectors using mosquito repellents or nets, and to move pigs from human settlements, although this can be logistically impractical (Fischer *et al.*, 2008). The effects of insecticide-treated mosquito nets on JEV seroconversion in pigs and humans was investigated by Dutta *et al.* (2011) in four comparable areas in Assam, north-eastern India. Each area was either provided with mosquito nets for both humans and pigs, only humans, only pigs, or left without nets. Blood was sampled from children and pigs and serologically tested for JEV. It was found that the use of nets covering pigs at night together with the use of nets covering beds during the time of sleeping led to a marked reduction in both pig and human infection, compared with the use of nets for either pigs or humans. Lastly, Mwandawiro *et al.* (2000), who found that mosquito vectors have an innate tendency to feed on cattle and that host preference is reinforced by feeding behaviour, suggest that increasing the availability of cattle may divert mosquitos from pigs, and thus lower the risk for JEV infection. However, none of the strategies mentioned above deal with the fact that pigs are not the only amplifying hosts; JEV is also transmitted from birds (Constable *et al*., 2017).

Although JEV mosquito vectors breed in various sources of standing water such as marches, flooded ditches and water-filled containers, their preferred larval habitat is rice fields (Keiser *et al.*, 2005). Several strategies have been proposed to reduce mosquito breeding in this habitat, such as chemical control with synthetic larvicides and insecticides, biologic control with larvicidal bacterial toxins, nematodes, fungi and larvivorous fish, and environmental control through alternate wet and dry irrigation (AWDI). In the latter, soil is dried out regularly, which impede the development of larvae into adult mosquitos. If this strategy is maintained throughout the entire cropping season, the number of larvae may be significantly reduced. Exposure of humans to mosquitos can be prevented using mosquito repellents, protective clothing and mosquito nets, but in endemic areas where JEV transmission is year-round, consistent use by the local population may be hard to achieve (Fischer *et al*., 2008).

Pig production in Cambodia

In 2018, Cambodia was estimated to have 1.76 million pigs (FAO, 2020b) and produce 86,500 tonnes of pork (FAO, 2020c). The gross production value of pork produced in 2016 was almost 750 million USD (FAO, 2018a), which represented 3.8% of Cambodia's gross domestic product that year (FAO, 2019b). In 2013, pork was the most consumed type of meat in Cambodia (6.5 kg per person and year), followed by beef, and chicken (FAO, 2018b). The majority of pigs are raised in the provinces around Phnom Penh, where the demand for pork is high (Knips, 2004). The pigs are a source of income as well as food for the farmers, although most pigs are sent to slaughter, and pork is rarely purchased back. Pig raising is often part of an integrated farming system, where rice cultivation is central (Samkol *et al*., 2008).

There are three systems of pig production in Cambodia: traditional small-scale pig raising, medium-scale pig production and intensive large-scale pig production (Huynh *et al.*, 2006). The first system is most common and is predominately found in rural areas, where pigs often roam freely (Samkol *et al*., 2008). Generally, two to four pigs are fattened during a period of 8 to 12 months to reach slaughter weight (Huynh *et al.*, 2006). More pigs are raised after the rice harvest season due to increased availability of rice by-products, which are used as feed. The most common feed is rice bran, but rice grain, cooked with vegetables or water plants, is also used (Samkol *et al*., 2008). In addition to rice by-products, pigs are given kitchen waste and occasionally concentrates. The feed is often inadequate in terms of both quantity and quality, which results in low growth rate in pigs and few produced litters due to delayed onset of oestrous in post-weaning sows. Indigenous breeds, such as Kandol, Domrei, Hainam and Kampot, perform better than exotic breeds on this type of diet (Samkol *et al*., 2008), and were earlier very common, along with several types of crossbreeds (Huynh *et al.*, 2006). Although pig raising may function as a form of capital investment (Knips, 2004), it only contributes to a small part of the overall income for these farmers (Huynh *et al.*, 2006).

Medium-scale farmers generally keep 10 to 50 pigs of different categories, such as sows, piglets and fatteners (Huynh *et al.*, 2006). The pigs are usually exotic breeds and crossbreeds. Farmers pursue either fattening, piglet or farrow-to-finish production, or a combination of these (Ström *et al*., 2017). Many of the farmers are also rice millers or rice wine producers, which gives them access to rice by-products, although pigs are given commercial feed as well (Huynh *et al.*, 2006). The pigs are confined in pens and mainly kept for commercial purposes, often generating a large part of the family income (Ström *et al*., 2017). Disease outbreaks, expensive feed and low slaughter prices are perceived as major constraints by the farmers. Larger-scale pig production started increasing in the 2000s, predominately near population centres (Knips, 2004). The first few specialised large-scale pig farms were mainly located in Phnom Penh, where most of the breeding stock and piglets sold to medium-scale producers come from (Samkol *et al*., 2008). These large-scale farms are well managed and have a high productivity. The pigs are exclusively exotic breeds, such as Yorkshire, Landrace and Duroc, and their feed consist of cereal grains, soybean meal and fish meal.

Circulation of JEV among Cambodian pigs

The first extensive study on JEV transmission in Cambodia, at the time suspected as highly endemic, was performed by Duong *et al*. (2011). In February and July 2006, a total of 393 samples were collected in Kampong Cham and Kampong Speu, respectively. In addition, 112 pigs from Kampong Cham, Kampong Speu and six other provinces were sampled at a slaughterhouse in Phnom Penh in December 2007. The rate of seropositivity was very high in samples from pigs originating in Prey Veng (100%), Svay Reang (100%), Kampot (100%), Pursat (96.3%) and Kandal (96%), and slightly lower in Kampong Speu (81.7%), Takeo (60%) and Kampong Cham (52.5%). When pigs were stratified into four age groups (less or equal to two months, two to four months, four to six months, and older than six months), the following rates were found, respectively: 47.5%, 56.2%, 87.3%, and 95.2%. The mean antibody titre was significantly higher in pigs older than six months than the other age groups.

The intensity of JEV transmission in Ta Khmau city, Kandal province was studied by Cappelle *et al.* (2016), who monitored two cohorts of 15 pigs each. The pigs were sampled for blood from two to six months of age between either April and July 2014, or September 2014 and January 2015. The samples were tested for JEV antibodies and RNA. All but one pig seroconverted during the study period. Some pigs were still protected by maternal antibodies during the first couple of weeks, but all had become seronegative by the age of three months. JEV RNA was only found in one sample, belonging to a pig in the second cohort. The estimated force of infection was 0.032 per day in the first cohort, and 0.046 per day in the second cohort, which means that susceptible pigs had 3.2% or 4.6% probability of acquiring JEV infection each day. During the first and second study period, 6,692 and 4,386 mosquitos were captured in traps placed close to the pigs. The most abundant species was *Cx. tritaeniorhynchus*, followed by *Cx. gelidus* and *Cx. vishnui*. Around one percent of the captured mosquitoes were *Cx. quinquefasciatus.* Despite the high seroconversion rate in pigs, only one pool of *Cx. tritaeniorhynchus*, captured in September, contained JEV RNA. In relation to the total number of mosquitos captured during the study, the minimum infection rate was 0.13 per 1,000 female *Cx. tritaeniorhynchus* and 0.09 per 1,000 female mosquitos of all captured species.

A similar study was conducted by Di Francesco *et al.* (2018), who monitored two cohorts of 15 pigs each; one in Ta Khmau city and one in a rural part of Kandal. The pigs were born in April and sampled for blood between June and October 2015. The samples were tested for JEV antibodies and RNA. Maternal antibodies waned between 2 and 3.5 months of age. All pigs seroconverted between 2.5 and 6 months of age, and three pigs in each cohort tested positive for JEV RNA. In the peri-urban farm, the average age at infection was three months, compared to five months in the rural farm. The estimated force of infection was 0.061 per day in the peri-urban cohort, and 0.069 per day in the rural cohort. The delayed seroconversion in the rural cohort may be explained by a dilution of mosquito bites created by higher pig density, since the pigs were raised among 120 other pigs. Also, the vector abundance was higher in the peri-urban farm, while cattle density was higher in the rural farm. The estimates are higher than the ones previously made by Cappelle *et al.* (2016), which may reflect seasonal variations. In the previous study, pigs were monitored during periods ending or beginning at the onset or end of the rainy season, whereas Di Francesco *et al.* began the monitoring at the peak of the rainy season. The number of mosquitoes captured near the pigs increased from July to October in the peri-urban farm, and from July to September in the rural farm, where it subsequently deceased in October. The three most abundant species were *Cx. tritaeniorhynchus*, *Cx. gelidus*, and *Cx. vishnui*. Very few *Cx. quinquefasciatus* were captured.

Samples from two pigs (one in each cohort) and one pool of *Cx. tritaeniorhynchus* in the study of Cappelle *et al.* (2016) and six pigs (three in each cohort) in the study of Di Francesco *et al.* (2018), contained JEV RNA (Duong *et al.*, 2017a). Full genomes were obtained from two pigs (one in each cohort) in the study of Di Francesco *et al.*, the mosquito pool as well as two human JE cases in Ratanakiri and Prey Veng provinces. Sequencing of the other pig samples only generated partial sequences of NS3 or E genes, or, in one case, was not successful. Phylogenetic analysis revealed that all sequenced strains belonged to either GI-a or GI-b; the human strains belonged to one clade each, six of the pig strains belonged to clade GI-b and one belonged to clade GI-a, and the mosquito strain belonged to clade GI-a. The pig strain belonging to clade

GI-a and the mosquito strain came from samples collected in September 2014 in Ta Khmau by Cappelle *et al*. The human strain belonging to clade GI-a came from the patient in Prey Veng and was sampled in July 2015. All clade GI-b pig strains came from samples collected by Di Francesco *et al.* in every month between June and October in 2015 in both Ta Khmau and rural Kandal. The human strain belonging to clade GI-b came from the patient in Ratanakiri and was sampled in May 2013. Taken together, the results of the studies of Duong *et al.*, Cappelle *et al.* and Di Francesco *et al.* demonstrate the existence of JEV circulation between humans, pigs and *Cx. tritaeniorhynchus* in Cambodia, not only in rural areas, but also in peri-urban areas near Phnom Penh.

Reproductive disorders

If susceptible, pregnant gilts or sows become infected with JEV during the first 60 to 70 days of gestation, it can cause reproductive disorders such as abortions, stillbirths and foetal mummification (Williams *et al.,* 2019). In endemic areas where transmission is year-round, most pigs become immune before reaching sexual maturity (Duong *et al*., 2011; Cappelle *et al.*, 2016; Di Francesco *et al.*, 2018). The immunity lasts for up to three years (Geevarghese *et al.*, 1994). Most breeding sows in endemic areas are therefore likely to be protected from JEV-induced reproductive disorders. Previous studies have generally, and quite understandably, focused on the effects on reproductive performance in epidemic areas where transmission is seasonal. The situation in Cambodia has not been investigated, but Lindahl *et al.* (2012b) performed a study in southern Vietnam, an endemic area with tropical climate, in which 315 sows from four state-owned pig farms located in the Mekong delta were sampled for JEV antibody detection, while information on reproductive performance was obtained from the farm managers. The rate of seropositive sows was 60%, and seropositivity was significantly correlated with age. Compared to sows under 1.5 years of age, sows older than 3.5 years had an odds ratio (OR) of 6.4 for being seropositive. Abortion was only observed in four sows, and all of them were seropositive. Only nine sows had more than two mummified foetuses, and no sow had more than five. In sows less than 1.5 years old, serologic status and breed were associated with the number of stillborn piglets when optical density (OD) values were continuous. However, there was no such association when seropositivity as determined by a fixed OD value was used as variable. No association between the number of stillborn piglets and seropositivity was found when sows of all ages were included in the analysis. The results suggest that JEV infection only has a negative impact on reproductive performance in young sows in areas with endemic transmission.

MATERIAL AND METHODS

Study area and population

In October 2019, rural, pig-raising smallholders were visited in Kampong Thom, Preah Vihear, Ratanakiri, and Stung Treng provinces, north-eastern Cambodia (see Figure 1). The study purposely selected provinces identified by the national authority to have the highest numbers of extensively kept smallholder pigs. In each province, three to four pig-abundant, geographically dispersed districts were selected by the provincial veterinarian. Households that met the inclusion criteria of keeping up to 10 pigs over three months of age were identified and selected by the village heads within the districts. All smallholders were informed about the study and asked for their consent to participate before the field worked commenced. In each household, one to three pigs over three months of age (to avoid interference of maternal antibodies) were sampled for blood. When possible, pigs of various ages were selected, including at least one sow. Pregnant sows and pigs that showed any signs of illness were excluded to avoid stressing the animals. A total number of 139 households and 242 pigs were included in the study.

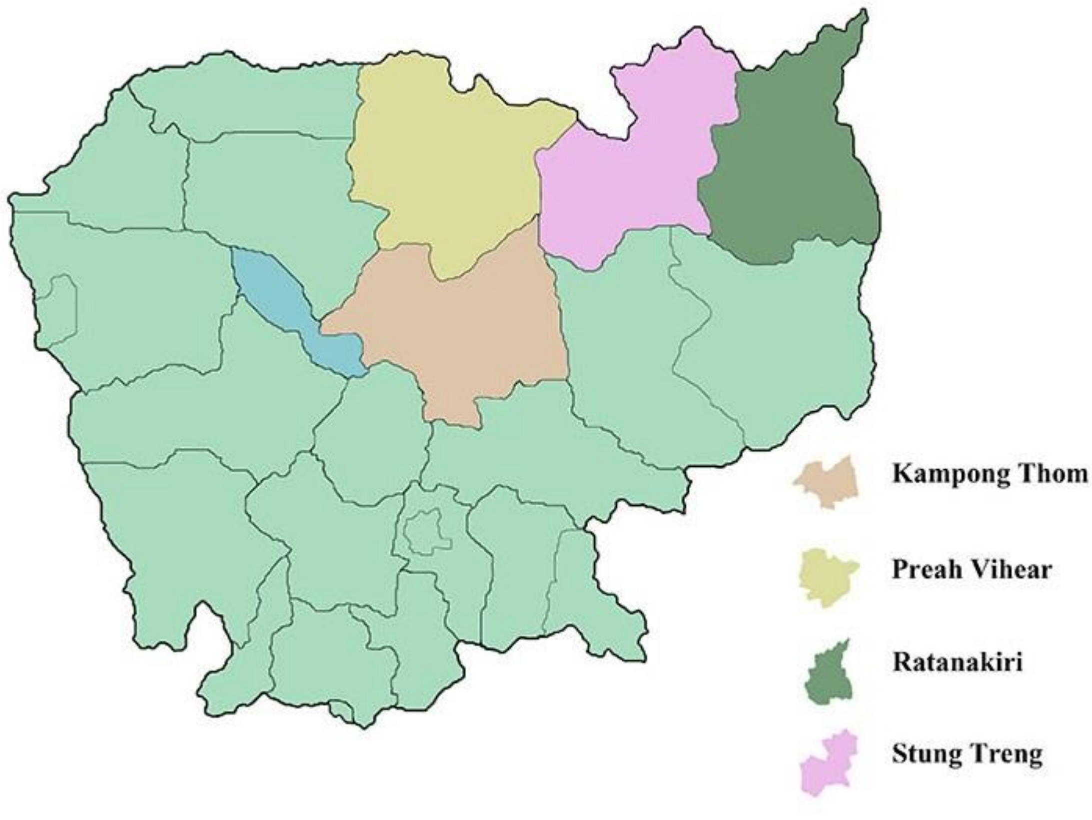

Figure 1. *Map over Cambodia and the studied provinces.*

Blood sample and data collection

Blood was sampled from the jugular or ear vein of the pigs into Vacuette serum tubes, which were individually labelled and kept in a cooling box. The samples were centrifuged within 24 hours and sera were transferred to labelled cryotubes and stored in a freezer (-18°C). During transportation to the laboratory at National Animal Health and Production Research Institute (NAHPRI) in Phnom Penh, the samples were kept in a cooling box. For each sampled pig, a form was filled out (see Appendix 1), including information about sex, age, breed and history of reproductive disorders. One adult person (over 15 years old) responsible for the pigs in each household was interviewed about pig management, occurrence of reproductive disorders, knowledge of JE and mosquito-borne diseases in general, and disease prevention. The questions were part of a questionnaire (see Appendix 2), which had been translated from English to Khmer by a Cambodian veterinary student, who conducted the interviews together with two fellow veterinary students. The questionnaire was quantitative but contained both open and closed questions.

Serological analysis

The samples were analysed in the laboratory of NAHPRI, Phnom Penh in the beginning of November 2019 with help from the laboratory staff and local veterinary students. Presence of JEV antibodies in sera was tested through competitive enzyme-linked immunosorbent assay (ELISA). Commercial *ID Screen® West Nile Competition Multi-species* kits (IDvet, Grabels), designed to detect antibodies against the envelope protein of West Nile virus (WNV) were used. Despite being designed for WNV and the risk of cross-reaction with antibodies against flaviviruses such as Dengue and Zika viruses, this product has been successful in detecting JEV specific antibodies in pig sera in northern Vietnam (Ruget *et al.,* 2018). However, there are no known sensitivity or specificity values for the ELISA when testing for JEV. The kits were used and interpreted according to the manufacturer's instruction, defining seropositivity in the test as JEV. All tests were validated against positive and negative test controls.

Data analyses

Descriptive and statistical analyses of data collected through the questionnaire, blood sample form and serological analysis of samples were made in Microsoft® Excel®, Office 365MSO and Stata® 14.2 (StataCorp Ltd, College Station, Texas). The true seroprevalence could not be estimated since there are no known sensitivity or specificity values for the ELISA. Thus, only the apparent seroprevalence was calculated. The significance of potential associations between serologic status in individual pigs or of households and other factors such as age and reproductive disorders was determined through T-test (age) and Fisher's Exact Test (age and other factors). Seropositive households were defined as households keeping one or more seropositive pigs. Associations between disease knowledge and gender or education of the respondent, and between vaccinated households and province, were also investigated, using Fisher's Exact Test.

RESULTS

Unfortunately, the test results of one ELISA plate with 46 samples from Preah Vihear could not be interpreted, since the OD values of the positive and negative test controls were similar. Only the results of three clearly negative samples were kept for data analyses.

Household and sample distribution

In Kampong Thom, Preah Vihear, Ratanakiri and Stung Treng, 39, 34, 35 and 31 households, respectively, answered the questionnaire, and 63, 51, 63 and 65 pigs, respectively, were sampled for blood (see Figure 2). Of the collected samples, most generated valid test results, but in Preah Vihear, only eight samples could be interpreted. One sample corresponds to one pig.

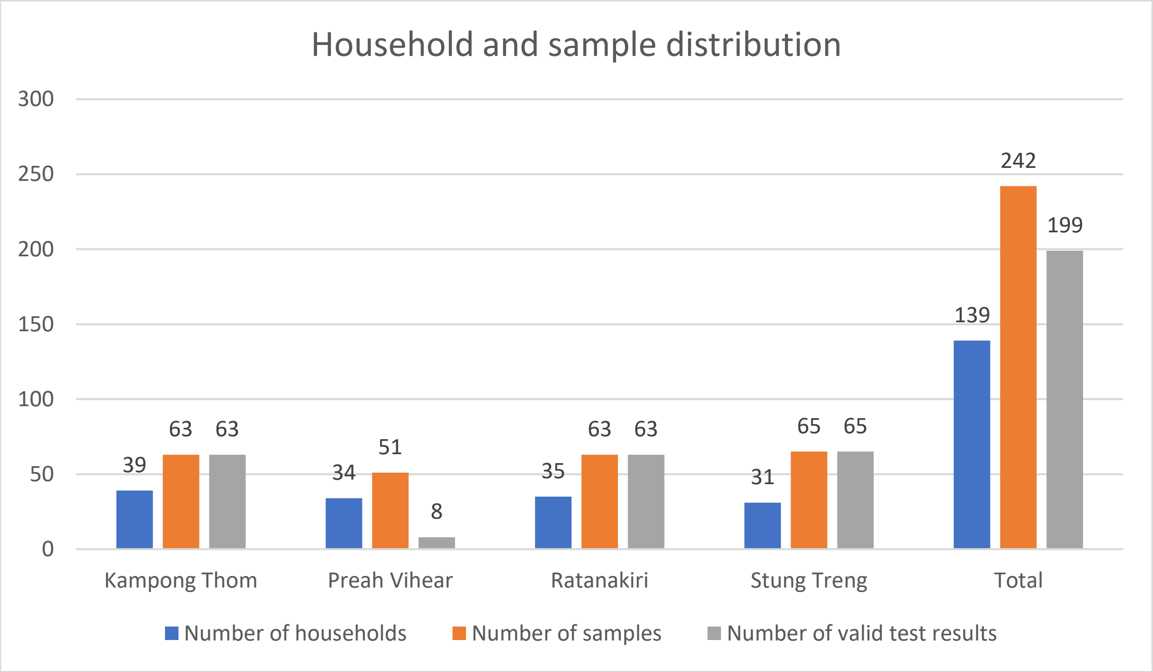

Figure 2. *Distribution of households, samples and valid test results within the provinces.*

The sampled pigs were between three months and five years of age, the mean age being nine months. The majority (60.9%) were female. In Preah Vihear and Stung Treng, over 90% of the pigs were indigenous breeds compared to Kampong Thom and Ratanakiri, where around half of the pigs were indigenous breeds (see Figure 3). The rest were mainly exotic breeds; very few were crossbreeds.

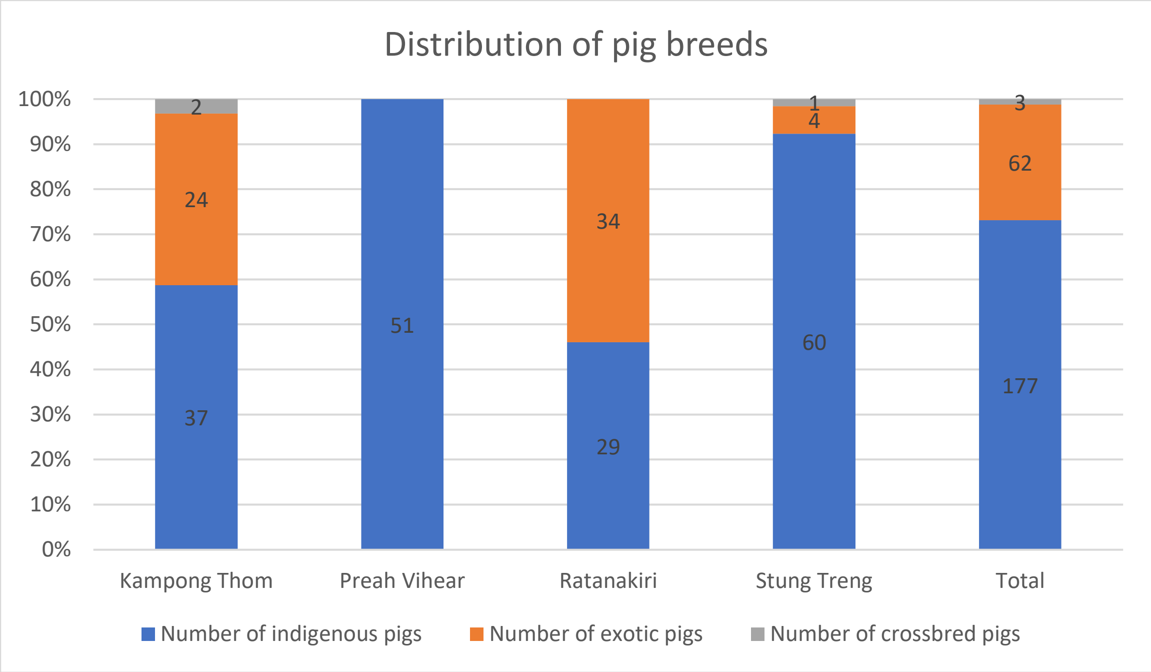

Figure 3. *Distribution of pig breeds within the provinces (numbers are indicated as numerals in bars).*

Household demographics

Of the respondents answering the questionnaire, 75.4% were female and 24.6% were male. The mean age was 41 years, ranging from 18 to 74. Most had finished primary school (42.6%), but 34.6% had no school education at all, and only one had a college or university degree. The share of uneducated respondents was highest in Kampong Thom (48.7%) and lowest in Stung Treng (12.9%).

Farm characteristics

On average, each household kept 4.2 pigs, ranging from 1 to 20. The distribution of pigs between different production categories can be seen in Table 1. Piglets are defined as pigs under one month of age, growers as pigs between one and three months of age, and fatteners as pigs over three months of age. Confinement in pens was the most common type of housing for the pigs; 80.6% of the households kept pigs confined, 40.3% kept pigs tethered, and 39.6% let pigs roam freely. Of the households keeping one or more sows, 79.4% kept them confined. The majority (54.2%) of households that let their pigs roam freely, did so during the dry season, compared to 12.5% during the rainy season, and 33.3% during both seasons.

Table 1. *Number of pigs in different production categories per household*

Production category	Mean number of pigs per household	Range
Piglets	1.0	(0–12)
Growers	1.4	(0–9)
Fatteners	1.1	(0–16)
Sows	0.6	(0–3)
Boars	0.1	(0–2)
Total	4.2	(1–20)

Seroprevalence

In total, 185 (93.0%) of the 199 samples that generated valid test results were positive (see Table 2). The age was not recorded for two of the seropositive pigs, which is why only 197 pigs are included in the age analysis. All pigs over six months of age had detectable JEV antibodies, compared to 89.1% of pigs between three and five months of age. The association between increasing age and seropositivity tended to be statistically significant (p=0.087). There was a highly significant difference in seroprevalence between the provinces (p<0.001), which was highest in Stung Treng (98.5%) and lowest in Preah Vihear (62.5%). The mean age of sero-positive pigs was 8.6 months (95% confidence interval 3.5–4.5), compared to 4 months (95% confidence interval 7.2–10.1) in seronegative pigs. Again, age tended to be significantly correlated with serologic status (p=0.080).

Table 2. *Seroprevalence of JEV antibodies among pigs of different age categories from all provinces*

Age category (p=0.087)	Number of seropositive pigs	Total number of pigs	%
3–5 months	114	128	89.1
6–11 months	31	31	100
≥12 months	38	38	100
Total	183	197	92.9
Province (p<0.001)			
Kampong Thom	62	63	98.4
Preah Vihear	5	8	62.5
Ratanakiri	54	63	85.7
Stung Treng	64	65	98.5
Total	185	199	93.0

All pigs that were crossbreeds tested positive, as did the vast majority of the exotic (96.8%) and indigenous (91.0%) pigs. Breed was not significant in regard to seropositivity (p=0.384). Of the households that let their pigs roam freely during the dry season, the rainy season or both seasons, 92.3% had one or more pigs that were seropositive, compared to 94.1% of the households that always kept their pigs confined or tethered. Almost all (90.9%) households that were in sight of rice fields were seropositive, compared to 97.4% of other households. Neither housing system (p=0.668) nor proximity to rice fields (p=0.213) were significantly associated with serologic status of households.

Reproductive disorders

No abortions had been observed in the sampled sows during the past year. Only one sow was known to have had both stillborn and mummified foetuses, two to have had stillborn foetuses, and one to have had mummified foetuses during this time. Test results were only valid for one of these sows, which was positive. When household representatives were asked if any of their sows, sampled or not, had aborted during the past year, 24.4% answered Yes. Stillborn or mummified foetuses were observed in 18.5% and 10.1% of households, while 9.3% and 11.2% had had weak or shaking piglets. There was no significant relationship between abortions reported for households and serologic status of households (p=0.336), although it is noteworthy that none of the seronegative households had experienced any abortions. Among the seropositive households, 22.9% had experienced abortions. Furthermore, no seronegative households had observed any stillborn or mummified foetuses, whereas 31.5% of the seropositive households had observed either, but there was no significance to this (p=0.519). Weak or shaking piglets were never observed in seronegative households but were reported by 13.0% of the seropositive households. In similarity with abortions, stillbirths, and mummified foetuses, there was no significant correlation between either weak (p=1.000) or shaking (p=1.000) piglets and serologic status of households, despite the absence of such piglets in seronegative households.

Disease knowledge and prevention

Of all 139 respondents, 98 (70.5%) had heard of Japanese encephalitis. Twenty-one (21.4%) of them claimed that they knew what it is, but only six delivered an explanation by describing clinical signs, such as fever, headache, diarrhoea and salivation. Of those who had heard of JE, 93 (94.9%) knew that humans can become infected, but only seven (7.1%) knew that pigs are susceptible. Only six (6.5%) of those who knew that people can become infected claimed that they knew how. Three delivered an explanation, of which only one – “from mosquitos” – was correct. Of the respondents who knew that pigs can become infected, only one claimed that he knew how, but gave no explanation. Four respondents had somebody in their family who had had JE, and four knew people outside of the family who had had it. One respondent reported that there had been one JE-caused death in the village. There were no significant relationships between disease knowledge (respondents who had heard of JE) and gender (male versus female, p=0.661) or education (any level of education versus no education, p=0.316) of the respondent.

When respondents that had heard of JE were asked if their family was vaccinated, all replied but one. In eight (8.2%) of the answering households, the whole family was vaccinated against JE, whereas in 85 (87.6%) of the households, only the children were vaccinated. In total, 93 (95.9%) households had vaccinated children. Of these households, 84 (90.3%) replied that the vaccinations had been funded by the government (there were no recorded answers for the other households). When households that were not vaccinated were asked why, one respondent said that they did not know that people can become infected with JEV, and one answered that they were afraid of vaccine side effects. The distribution of vaccinated households within the provinces can be seen in Figure 4. There was no significant correlation between vaccinated households (any households with vaccinated family members) and province (*p*=0.149), but for households were all family members were vaccinated, the difference between the provinces was significant (*p*=0.002). In Preah Vihear and Stung Treng, the whole family was vaccinated in 27.3% and 6.9% of the households, and in Kampong Thom and Ratanakiri, there were no such households.

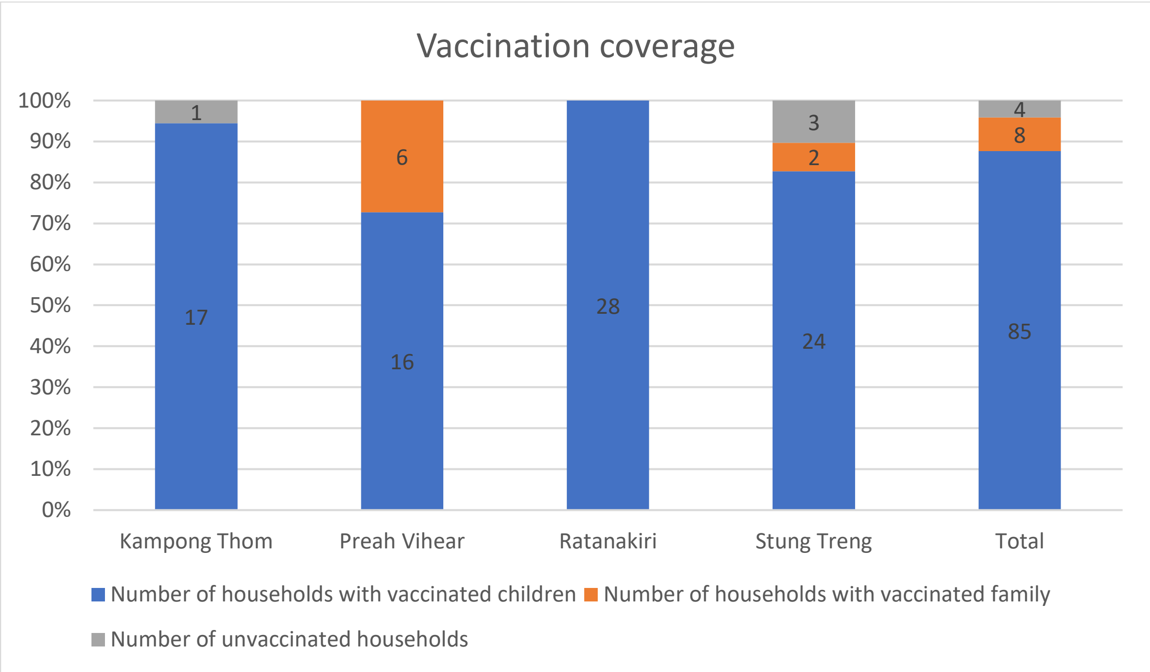

Figure 4. *Vaccination coverage in the studied provinces (numbers are indicated as numerals in bars).*

Of 137 respondents, 134 (97.8%) had heard of diseases being transmitted to people through mosquito bites. Although it was not included as a question, 23.9% of them mentioned Malaria, 9.0% mentioned Dengue, and 11.2% knew that mosquito-borne diseases can be serious. All 139 households used some sort of mosquito protection: 96.4% used bed nets, 56.8% used insect repellents, and 5.8% used covering clothes. Twenty-one (15.1%) households protected their pigs in some way. Of these households, 47.6% used insect repellents, 14.3% used smoke, one (4.8%) used mosquito nets, and one used light. There was no significant relationship between protection of pigs and serologic status of households (*p*=1.000).

DISCUSSION

The apparent prevalence of JEV antibodies observed in this study was high, as all pigs over six months of age (the oldest being five years old) and 89.1% of pigs from three to six months of age tested positive with the ELISA. The high seroprevalence as well as the slight difference between these age groups was rather expected, when comparing the results with results of other studies on JEV infection in pigs in Cambodia. In the study of Duong *et al.* (2011), 95.2% of pigs between six months and one year of age were positive, compared to 87.3% of pigs between four and six months of age. Taken together, in the studies of Cappelle *et al.* (2016) and Di Francesco *et al.* (2018), 98.3% of the monitored pigs seroconverted before six months of age. The reason for the lower prevalence in younger pigs is most likely explained by the weaning of maternal antibodies around three months of age followed by the varying, although relatively high force of infection in susceptible pigs, as observed by Cappelle *et al.* (2016) and Di Francesco *et al.* (2018). The ELISA test does not distinguish between actively produced and passively acquired JEV antibodies. Therefore, it cannot be assumed that all positive pigs under six months of age have been infected with JEV. Some are probably positive because they still are protected by maternal antibodies. In the study of Scherer *et al.* (1959), most pigs lost their passive immunity between four and six months of age, but for some, maternal antibodies were still detectable at seven months of age.

The difference of seroprevalence between provinces was statistically significant in this study ($p<0.001$). However, for Preah Vihear, where the prevalence (62.5%) was remarkably lower than in the other provinces, 43 of the samples could not be interpreted and were thus excluded from the data analyses. Only eight samples remained, of which five were positive. There is a high probability that the low number of included samples may not be representative for the whole province. Duong *et al.* (2011) found that the prevalence varied between the eight provinces included in their study. In three of the studied provinces, all pigs between two months and one year of age were positive, compared to 52.5% in Kampong Cham and 60% in Takeo. A large number of sera (393) were collected in Kampong Cham and Kampong Speu, although there is no further information about the source population. However, because of the large sample size and the low apparent prevalence in Kampong Cham, a low true prevalence in Preah Vihear cannot be ruled out. In the future, it would be interesting to investigate why the prevalence is apparently lower in these provinces.

A possible source of false positive test results in this study is the unknown specificity of the ELISA test. It is designed to detect antibodies against the envelope protein of West Nile virus, but cross-reacts with antibodies against other flaviviruses, which exhibit the same type of protein. The test has been successfully used to detect JEV antibodies in sera from pigs in northern Vietnam (Ruget *et al.*, 2018). In that study, positive samples were confirmed with virus neutralization test (VNT), which is a highly specific serological assay used to distinguish between antibodies against different flaviviruses. Except for JEV, the only flaviviruses present in Cambodia and Vietnam that can infect and induce antibody production in pigs are Dengue and Zika viruses (Huy *et al.*, 2010; Cuong *et al.*, 2011; Chu *et al.*, 2017; Duong *et al.*, 2017b; Williams *et al.*, 2019). However, infection with these viruses have only been observed in experimentally infected pigs (Williams *et al.*, 2019), and the circulation of Zika virus is low in Cambodia (Duong *et al.*, 2017b). Dengue is highly endemic in Cambodia and clinical cases occur year-round, although more cases are reported during the rainy season (Huy *et al.*, 2010). Therefore, antibodies against Dengue virus could potentially have been present in the tested sera in this study. In the study of Ruget *et al.* (2018), blood samples were collected from pigs at a slaughterhouse in Hanoi, originating in surrounding areas of the Red River delta, between October 2009 and September 2010. Although the transmission of Dengue virus is low in Hanoi, the incidence of clinical cases has increased since 1999 (Cuong *et al.*, 2011). Moreover, there was a large outbreak in 2009, concurring with the blood sampling by Ruget *et al.* (2018). Because specific JEV antibodies were successfully detected with the ELISA, despite the ongoing circulation of Dengue virus in the area, it seems unlikely that the positive test results in the current study would represent Dengue virus and not JEV infections. The predictive value of a positive test (PVPT) could not be calculated since there are no known sensitivity or specificity values for the ELISA. However, since the PVPT correlates with prevalence, and the apparent prevalence was very high, most of the positive rest results are likely to be true positive.

There were no significant associations between reproductive disorders and serologic status of households, but abortions, stillbirths, mummified foetuses and weak or shaking piglets had not been observed in any of the seronegative farms during the past year. Reproductive disorders had not been observed in most seropositive households either, but the total absence in all seronegative households is still an interesting finding. However, as all pigs over six months of age were seropositive, the presence of seronegative pigs in seronegative households is not an indication of the serological status in adult, sexually mature pigs. Given the results of this study, these pigs are likely to be seropositive. Reproductive disorders occur if susceptible gilts or sows become infected during the first 60 to 70 days of gestation (Williams *et al.*, 2019). In this study, all pigs over six months of age were immune at the time of sampling, including those under one year of age. Most of them had probably become infected before reaching sexual maturity. Therefore, JEV-induced reproductive disorders is not likely to be an issue in Cambodia, as adult pigs are protected through early infection and immunization.

Households were asked if they had observed any reproductive disorders during the past year. This is a long time period and it may have been difficult for the respondents to remember. Recall bias can influence the outcomes of a study and be a potential threat to the internal validity (Coughlin, 1990). Moreover, many pigs had been stamped during the outbreaks of African swine fever that hit Cambodia and many other Asian countries in the beginning of 2019. During the household visits, the provincial official veterinarian was always attendant, and respondents might have been cautious to mention if their pigs had showed any signs of disease. Additionally, there are other etiologies of reproductive disorders in sows, and these were not taken into consideration. However, most households – both seropositive and seronegative – had not observed any reproductive disorders. Thus, this is not as confounding as if there had been an observed difference between the groups.

Almost all respondents had heard of mosquito-borne diseases, and over two-thirds had heard of JE. However, only one person knew that people become infected with JEV through mosquito bites. This question was asked as an open question without any pre-written alternative answers. For most households, there was no recorded answer, and it is impossible to tell whether the respondents were asked this question or not. Also, it seems like there were misinterpretations of this question, as one respondent answered by describing symptoms of the disease and one respondent replied that there had been one JE-caused death in the village. There were also other questions for which recorded answers were missing. For example, 21.4% of the respondents who had heard of JE answered Yes to the question “Can you explain what it is?”, but only 28.6% of them did in fact explain. The questionnaire was translated from English to Khmer, the official language in Cambodia, and the answers were translated back to English. It is possible that the language barrier caused some misinterpretations during this process. The interviews were conducted by three Cambodian veterinary students. One of the students interviewed all households in Ratanakiri and Stung Treng and some of the households in Kampong Thom and Preah Vihear, where either of the other two students interviewed the remaining households. The students may have interpreted the questions and answers differently, which can have reduced the consistency of the results.

Although the knowledge of JEV transmission appeared to be low, most respondents had heard of mosquito-borne diseases, and all households used some type of mosquito protection. Almost all household used bed nets, which assumingly are fixed to the interior and used every night. More than half of the households used insect repellent, but there was no follow-up question about how frequently it was applied. In retrospective, this had been interesting to know. Because almost all respondents knew that mosquito bites can cause disease, they were probably motivated to protect themselves, and if insect repellents were not applied frequently enough to provide protection, it must have had other causes, such as practical inconvenience or high costs.

Only one household used mosquito nets to protect their pigs, while 10 used insect repellents and three used smoke. Although the use of bed nets led to a reduction of JEV infections in humans in the study of Dutta *et al.* (2011), the combined use of bed nets for humans and mosquito nets for pigs was more successful. Households that did not protect their pigs were not asked why, but one possible reason is that they do not know that pigs can become infected through mosquito bites. Because JEV infection usually is subclinical in pigs (Williams *et al.*, 2019), it is understandable if pig raisers are not aware of the infection risk. Only one respondent replied that pigs can become infected with JEV but could not explain how. It is possible that this person guessed the right answer. Furthermore, it is even less likely that pig raisers are informed about the circulation of JEV between pigs and humans. The effects of mosquito protection for pigs would need to be evaluated in the ecological setting it is intended for, since there are many factors that influence the intensity of JEV transmission, such as precipitation (Murty *et al.*, 2010), mosquito breeding grounds (Keiser *et al.,* 2005) and amplifying birds (Constable *et al*., 2017), and there may be other preventive measures that are more favourable. In this study, there was no observed difference between the households that protected their pigs and the other households regarding serologic status. It is difficult to tell why, since the use of mosquito protection was not further investigated.

One proposed strategy to reduce the transmission of JEV is to vaccinate pigs. However, there are many reasons for why this strategy would not be applicable to the Cambodian context. Firstly, which is a general liability, the turn-over rate in pig production is high, and vaccination would need to be performed frequently and to high costs in terms of both management and money (Williams *et al.*, 2019). Secondly, because most adult sows in Cambodia are likely to be immune to JEV, as observed in this study and by Duong *et al.* (2011), piglets acquire passive immunity through colostrum intake. The presence of maternal antibodies could interfere with vaccination, making it ineffective (Williams *et al.*, 2019). Thirdly, vaccination of pigs does not eliminate the risk of human infection, since JEV can also be transmitted from birds (van den Hurk *et al.*, 2009). Many households in Cambodia keep chickens or ducks (Knips, 2004; Di Francesco *et al*., 2018), which have potential to infect mosquito vectors with JEV (Cleton *et al.*, 2014). Moreover, wild reservoir birds such as herons and egrets (van den Hurk *et al.*, 2009) are present in the country.

Another proposed control strategy is to reduce the numbers of mosquito vectors by reducing the number of larvae in rice fields. Keiser *et al.* (2005) compared chemical and biological intervention strategies and concluded that one of the most sustainable and worthwhile strategies was alternate wet and dry irrigation (AWDI). However, this strategy can only be performed in settings where irrigation water can be managed. The irrigation infrastructure in Cambodia has grown gradually since the 1990s but remains significantly underdeveloped (FAO, 2011). In 2010, approximately 24% of the rice land was estimated to be irrigated. Most rice fields are rain fed, with or without supplementary irrigation. Drainage systems are poorly developed, and floods are frequent during the rainy season. Because of limited irrigation and drainage possibilities, AWDI may not be very feasible in Cambodia. If the water supply and drainage of rice fields could be controlled, the number of mosquito vectors could potentially be reduced.

Contrarily to what was expected, there was no correlation between proximity to rice fields in this study, which is the preferred habitat of the major mosquito vectors (Keiser *et al.*, 2005), and serologic status of households. In fact, the percentage of seropositive households was lower for households that were within sight of rice fields (90.9%) than for other households (97.4%). One possible explanation for this result could be that other households really *were* in proximity of rice fields, and that other houses blocked the view. It would then have been more accurate to look around for rice fields within the neighbourhood. More likely, it was not possible to distinguish any factors associated with serologic status because of the high seroprevalence in this study.

Only the households that had heard of JE (which represented two-thirds of all households) were asked whether they were vaccinated against JE or not. Almost all of these households had vaccinated children, and over 90% of the vaccinations were funded by the government. The households that had not heard of JE had probably not heard of it because they were not vaccinated or were vaccinated but did not know against what. There was no significant difference between the provinces regarding the prevalence of households with vaccinated children. Therefore, when looking at the results for the answering households, the vaccinations coverage in children appears to be high in all provinces. The incorporation of JE into the national childhood immunization program in 2016 (Heffelfinger *et al*., 2017) must have been successful in reaching children of rural families in these areas.

CONCLUSIONS

The results of this study confirm that JEV indeed is endemic in Cambodia, and that most pigs become infected before reaching sexual maturity. Thus, their reproductive performance is not affected. No risk factors for infection apart from province could be identified because of the high seroprevalence in the study population. The seroprevalence was relatively low for the pigs in Preah Vihear but is not necessarily representative for the whole province, since very few test results were valid. All families used mosquito protection, and almost all respondents had heard of mosquito-borne diseases. Most respondents had heard of JE, but only one could explain how people become infected. Less than one in five households protected their pigs from mosquito bites. The vaccination coverage in children of rural families in Kampong Thom, Preah Vihear, Ratanakiri and Stung Treng appears to be satisfactory. However, national vaccination should be continued to prevent future cases of JE in Cambodia, where JEV transmission is highly intense, as observed in this study.

ACKNOWLEDGEMENTS

This study was performed as a Minor Field Study project through a scholarship from the Swedish International Development Cooperation Agency (Sida). Additional funding was received from the Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences (SLU); the Cambodian Safe Food Fair Food project, International Livestock Research Institute (ILRI); and the Metropolitan Mosquito project. I would also like to thank my Swedish supervisors Gunilla Ström Hallenberg and Johanna Lindahl; Dr. Sothyra Tum, Director of National Animal Health and Production Research Institute (NAHPRI), Cambodia; the local team of field and lab workers; and participating households. Most importantly, I would like to acknowledge my brave friend and study partner Rebecca Söderberg, who made it possible to conclude our shared project.

POPULAR SCIENCE SUMMARY

Japanese encephalitis (JE) is a neurologic disease, caused by Japanese encephalitis virus (JEV), which infects and causes inflammation of the brain. There have been several JE outbreaks in many Asian countries during the last century, since the first cases were reported in 1924 in Japan. Today, JE exists in 24 Asian and Western Pacific countries, which are home to around half of the world's population. Almost 68,000 cases occur annually, although the numbers have decreased significantly because of the establishment of childhood vaccination programmes in many countries. Most people who become infected do not show any – or only mild – symptoms of disease. Among those who develop severe symptoms, around 20–30% die, and up to half of the survivors are faced with permanent disabilities. In Cambodia, cases of JE occur year-round, often in children. Sub-national vaccination of children was initiated in 2009, after two years of hospital surveillance confirmed JE as a major health concern in the country. In 2016, a national vaccination programme was established.

The virus is transmitted through bites from infected mosquito vectors, the major vector species being mosquitos within the *Culex vishnui* subgroup. It can infect several vertebrate species, but most species do not show any signs of disease. Humans, as well as most animal host species, do not contribute to further transmission of the virus. Among wild animals, water birds such as herons and egrets are considered the most important virus-preserving hosts for JEV; they develop high levels of viruses that circulate in their blood and infect feeding mosquitos, without showing any signs of disease. Domestic pigs also serve as important preserving hosts. In similarity with wild water birds, they develop high virus levels, often without becoming ill. However, infection in adult pigs can cause reproductive disorders, such as abortions, stillbirths, mummified foetuses, weak piglets and infertility. Because the major vector species breed in rice fields and pigs can infect feeding mosquitos, JE is strongly associated with rice cultivation and pig rearing. The disease is mainly considered rural, although there is evidence of virus circulation in peri-urban and urban areas as well.

The purpose of this study was to determine how common it is that pigs kept by rural smallholders in Cambodia have been exposed to JEV by detecting antibodies against the virus in pig blood. The share of pigs that have become infected and developed JEV antibodies is called *seroprevalence*. The seroprevalence in pigs is indicative of how intense the JEV transmission is in these areas. By determining the prevalence, the importance of disease prevention in areas with similar characteristics can be stressed. Previously, the situation has only been investigated in the southern part of Cambodia, where the seroprevalence appears to be high, especially in adult pigs. Therefore, this study was set in Kampong Thom, Preah Vihear, Ratanakiri and Stung Treng provinces, north-eastern Cambodia, where many households pursue extensive pig rearing.

The seroprevalence was determined by analysing blood samples from 242 pigs in 139 households, evenly distributed between and within provinces. Only pigs over three months of age were sampled to avoid possible interference of maternal antibodies, which are obtained passively by piglets from the milk of immune sows. The maternal antibodies usually disappear around three months of age, and pigs become susceptible to infection. The observed (apparent) seroprevalence was 93.0% for all pigs, 89.1% for pigs between three and six months of age, and 100% for pigs over six months of age.

Another aim of the study was to investigate whether there are any risk factors for infection, such as breed, housing system and proximity to rice fields, and if serologic status (presence or absence of antibodies against JEV) is associated with reproductive disorders. Female pigs that still are susceptible when they reach sexual maturity may become infected while they are pregnant. The virus may then cross the placenta and infect the foetuses, causing foetal death – observed as abortions, stillbirths and mummified foetuses – or weakness in born, live piglets. Such production losses can have a major impact on the profitability of pig rearing.

Information about sex, age, breed and history of reproductive disorders was recorded for each sampled pig, and household representatives were asked questions about pig management and occurrence of reproductive disorders. Because of the high seroprevalence, no significant differences between positive and negative pigs were found, except province. The seroprevalence was highest in Stung Treng, where 98.5% of the pigs were positive, compared to 62.5% in Preah Vihear, where it was lowest. However, very few test results were valid for Preah Vihear, and thus, the prevalence is not necessarily representative for the whole province.

The household representatives were also asked questions about JE, mosquito-borne diseases in general and preventive measures, to find out how well-informed people are, if they protect themselves or their pigs from mosquito bites, and if children (or other household members) are vaccinated against JE. Almost all respondents knew that mosquitos can transmit diseases to humans, and over two-thirds had heard of JE. All households protected themselves from mosquito bites in some way; almost all used bed nets, and over half used insect repellents. Less than one in five households used mosquito protection for their pigs. The vaccination coverage in children appeared to be high in all provinces.

The results of this study suggest that JEV transmission is intense, not only in southern Cambodia, but in north-eastern, rural areas as well. Fortunately, people are well-aware of mosquito-borne diseases and protect themselves from mosquito bites, and many children are vaccinated against JE. Nonetheless, it is important that national vaccination is continued.

REFERENCES

Amerasinghe, F.P. & Indrajith, N.G. (1995). Nocturnal biting rhythms of mosquitoes (Diptera Culicidae) in Sri Lanka. *Tropical Zoology,* 8:43–53. doi:10.1080/03946975.1995.10539271

Bi, P., Tong, S., Donald, K., Parton, K.A. & Ni, J. (2003). Climate variability and transmission of Japanese encephalitis in eastern China. *Vector-Borne and Zoonotic Diseases*, 3(3):111–115.

Burke, D.S., Tingpalapong, M., Elwell, M.R., Paul, P.S. & Van Deusen, R.A. (1985). Japanese encephalitis virus immunoglobulin M antibodies in porcine sera. *American Journal of Veterinary Research*, 46(10):2054–2057.

Campbell, G.L., Hills, S.L., Fischer, M., Jacobson, J.A., Hoke, C.H., Hombach, J.M., Marfin, A.A., Solomon, T., Tsai, T.F., Tsu, V.D. & Ginsburg, A.S. (2011). Estimated global incidence of Japanese encephalitis: a systematic review. *Bulletin of the World Health Organization*, 89:766–774. doi:10.2471/BLT.10.085233

Cappelle, J., Duong, V., Pring, L., Kong, L., Yakovleff, M., Prasetyo, D.B., Peng, B., Choeung, R., Duboz, R., Ong, S., Sorn, S., Dussart, P., Tarantola, A., Buchy, P. & Chevalier, V. (2016). Intensive circulation of Japanese encephalitis virus in peri-urban sentinel pigs near Phnom Penh, Cambodia. *PLoS Neglected Tropical Diseases*, 10(12):e0005149. doi:10.1371/journal.pntd.0005149

Chu, D.-T., Ngoc, V.T.N. & Tao, Y. (2017). Zika virus infection in Vietnam: current epidemic, strain origin, spreading risk, and perspective. *European Journal of Clinical Microbiology and Infectious Diseases*, 36:2041–2042. doi:10.1007/s10096-017-3030-8

CIA (2020). *World fact book – Cambodia*. https://www.cia.gov/library/publications/the-world-factbook/geos/cb.html [2020-02-09]

Cleton, N.B., Bosco-Lauth, A., Page, M.J. & Bowen, R.A. (2014). Age-related susceptibility to Japanese encephalitis virus in domestic ducklings and chicks. *American Society of Tropical Medicine and Hygiene,* 90(2):242–246. doi:10.4269/ajtmh.13-0161

Constable, P.D., Hinchcliff, K.W., Done, S.H. & Grünberg, W. (eds.) (2017). Diseases of the nervous system. In: *Veterinary Medicine: A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs, and Goats*. 11. ed. St. Louis, Missouri, USA: Elsevier, 1155–1370. doi:10.1016/B978-0-7020-5246-0.00014-0

Coughlin, S.S. (1990). Recall bias in epidemiologic studies. *Journal of Clinical Epidemiology*, 43(1):87–91.

Cuong, H.Q., Hien, N.T., Duong, T.N., Phong, T.V., Cam, N.N., Farrar, J., Nam, V.S., Thai, K.T.D., & Horby, P. (2011). Quantifying the emergence of Dengue in Hanoi, Vietnam: 1998–2009. *PLoS Neglected Tropical Diseases*, 5(9):e1322. doi:10.1371/journal.pntd.0001322

Desingu, P.A., Ray, P.K., Patel, B.H.M., Singh, R., Singh, R.K. & Saikumar, G. (2016) Pathogenic and genotypic characterization of a Japanese encephalitis virus isolate associated with reproductive failure in an Indian pig herd. *PLoS ONE,* 11(2):e0147611. doi:10.1371/journal.pone.0147611

Diallo, A.O.II., Chevalier, V., Cappelle, J., Duong, V., Fontenille, D. & Duboz, R. (2018). How much does direct transmission between pigs contribute to Japanese encephalitis virus circulation? A modelling approach in Cambodia. *PLoS ONE*, 13(8):e0201209. doi:10.1371/journal.pone.0201209

Duong, V., Choeung, R., Gorman, C., Laurent, D., Crabol, Y., Mey, C., Peng, B., Di Francesco, J., Hul, V., Sothy, H., Santy, K., Richner, B., Pommier, J.-D., Sorn, S., Chevalier, V., Buchy, P., de Lamballerie, X., Cappelle, J., Horwood, P.F., & Dussart, P. (2017a). Isolation and full-genome sequences of Japanese encephalitis virus genotype I strains from Cambodian human patients, mosquitoes and pigs. *Journal of General Virology*, 98:2287–2296. doi:10.1099/jgv.0.000892

Duong, V., Ong, S., Leang, R., Huy, R., Ly, S., Mounier, U., Ou, T., In, S., Peng, B., Ken, S., Buchy, P., Tarantola, A., Horwood, P.F. & Dussart, P. (2017b). Low circulation of Zika virus, Cambodia, 2007–2016. *Emerging Infectious Diseases*, 23(2):296–299. doi:10.3201/eid2302.161432

Duong, V., Sorn, S., Holl, D., Rani, M., Deubel, V. & Buchy, P. (2011). Evidence of Japanese encephalitis virus infections in swine populations in 8 provinces of Cambodia: Implications for national Japanese encephalitis vaccination policy. *Acta Tropica*, 120:146–150. doi:10.1016/j.actatropica.2011.07.008

Dutta, P., Khan, S.A., Khan, A.M., Borah, J., Sarmah, C.K. & Mahanta, J. (2011). The effect of insecticide-treated mosquito nets (ITMNs) on Japanese encephalitis virus seroconversion in pigs and humans. *American Journal of Tropical Medicine and Hygiene*, 84(3):466–472. doi:10.4269/ajtmh.2011.10-0270

Erlanger, T. E., Weiss, S., Keiser, J., Utzinger, J. & Wiedenmayer, K. (2009). Past, present, and future of Japanese encephalitis. *Emerging Infectious Diseases*, 15(1):1–7. doi:10.3201/eid1501.080311

FAO (2011). *AQUASTAT Country profile – Cambodia*. Rome, Italy: Food and Agriculture Organization of the United Nations. http://www.fao.org/3/ca0391en/CA0391EN.pdf

FAO (2018a). *FAOSTAT – Value of agricultural production*. http://www.fao.org/faostat/en/#data/QV [2020-02-07]

FAO (2018b). *FAOSTAT – Food supply – Livestock and fish primary equivalent.* http://www.fao.org/faostat/en/#data/CL [2020-02-07]

FAO (2019a). *FAO STAT – Annual population.* http://www.fao.org/faostat/en/#data/OA [2020-02-07]

FAO (2019b). *FAOSTAT – Macro indicators*. http://www.fao.org/faostat/en/#data/MK [2020-02-07]

FAO (2020a). *FAO STAT – Crops.* http://www.fao.org/faostat/en/#data/QC [2020-02-07]

FAO (2020b). *FAOSTAT – Live animals.* http://www.fao.org/faostat/en/#data/QA [2020-02-07]

FAO (2020c). *FAOSTAT – Livestock primary.* http://www.fao.org/faostat/en/#data/QL [2020-02-07]

Fischer, M., Hills, S., Staples, E., Johnson, B., Yaich, M. & Solomon, T. (2008). Japanese encephalitis prevention and control: advances, challenges, and new initiatives. In: Scheld, W.M., Hammer, S.M. & Hughes, J.M. (eds.), *Emerging Infections 8*. Washington, D.C., USA: ASM Press, 93–124. doi:10.1128/9781555815592.ch6

Di Francesco, J., Choeung, R., Peng, B., Pring, L., Pang, S., Duboz, R., Ong, S., Sorn, S., Tarantola, A., Fontenille, D., Duong, V., Dussart, P., Chevalier, V. & Cappelle, J. (2018). Comparison of the dynamics of Japanese encephalitis virus circulation in sentinel pigs between a rural and a peri-urban setting in Cambodia. *PLoS Neglected Tropical Diseases*, 12(8):e0006644. doi:10.1371/journal.pntd.0006644

Gao, X., Liu, H., Li, X., Fu, S., Cao, L., Shao, N., Zhang, W., Wang, Q., Lu, Z., Lei, W., He, Y., Cao, Y., Wang, H. & Liang, G. (2019). Changing geographic distribution of Japanese encephalitis virus genotypes, 1935–2017. *Vector-Borne and Zoonotic Diseases*, 19(1):35–44. doi:10.1089/vbz.2018.2291

Geevarghese, G., Shaikh, B.H., Jacob, P.G. & Bhat, H.R. (1994). Persistence of haemagglutination-inhibition antibodies to JE and WN viruses in naturally infected domestic pigs in Karnataka State, India. *Acta Virologica*, 38(4):235–237.

Ghosh, D. & Basu, A. (2009). Japanese encephalitis – a pathological and clinical perspective. *PLoS Neglected Tropical Diseases*, 3(9):e437. doi:10.1371/journal.pntd.0000437

Gingrich, J.B. Nisalak, A., Latendresse, J.R., Sattabongkot, J., Hoke, C.H., Pomsdhit, J., Chantalakana, C., Satayaphanta, C., Uechiewcharnkit, K. & Innis, B.L. (1992). Japanese encephalitis virus in Bangkok: factors influencing vector infections in three suburban communities. *Journal of Medical Entomology*, 29(3):436–444.

Heffelfinger, J.D., Li, X., Batmunkh, N., Grabovac, V., Diorditsa, S., Liyanage, J.B., Pattamadilok, S., Bahl, S., Vannice, K.S., Hyde, T.B., Chu, S.Y., Fox, K.K., Hills, S.L. & Marfin, A.A. (2017). *Japanese encephalitis: surveillance and immunization in Asia and the Western Pacific, 2016.* Centers for Disease Control and Prevention, US Department of Health and Human Services. (Morbidity and Mortality Weekly Report, 66(22):579–583) doi:10.15585/mmwr.mm6622a3

Hills, S.L., Cuong, N.V., Touch, S., Mai, H.H., Soeung, S.C., Lien, T.T.H., Samnang, C., Sovann, L., Diu, P.V., Lac, L.D., Heng, S., Huong, V.M., Grundy, J.J., Huch, C., Lewthwaite, P., Solomon, T. & Jacobson, J.A. (2011). Disability from Japanese encephalitis in Cambodia and Viet Nam. *Journal of Tropical Pediatrics*, 57(4):241–244. doi:10.1093/tropej/fmp133

Hsu, S.M., Yen, A.M.F. & Chen, T.H.H (2008). The impact of climate on Japanese encephalitis. *Epidemiology and Infection*, 136:980–987. doi:10.1017/S0950268807009454

van den Hurk, A.F., Ritchie, S.A. & Mackenzie, J.S. (2009). Ecology and geographical expansion of Japanese encephalitis virus. *Annual Review of Entomology*, 54:17–35. doi:10.1146/annurev.ento.54.110807.090510

Huy, R., Buchy, P., Conan, A., Ngan, C., Ong, S., Ali, R., Duong, V., Yit, S., Ung, S., Te, V., Chroeung, N., Pheaktra, N.C., Uok, V. & Vong, S. (2010). National dengue surveillance in Cambodia 1980–2008: epidemiological and virological trends and the impact of vector control. *Bulletin of the World Health Organization*, 88:650–657. doi:10.2471/BLT.09.073908

Huynh, T.T.T., Aarnink, A.J.A., Drucker, A. & Verstegen, M.W.A. (2006). Pig production in Cambodia, Laos, Philippines, and Vietnam: a review. *Asian Journal of Agriculture and Development*, 4(1):69–90.

Keiser, J., Maltese, M.F., Erlanger, T.E., Bos, R., Tanner, M., Singer, B.H. & Utzinger, J. (2005). Effect of irrigated rice agriculture on Japanese encephalitis, including challenges and opportunities for integrated vector management. *Acta Tropica*, 95:40–57. doi:10.1016/j.actatropica.2005.04.012

Khan, S.U., Salje, H., Hannan, A., Islam, M.A., Bhuyan, A.A.M., Islam, M.A., Rahman, M.Z., Nahar, N., Hossain, M.J., Luby, S.P. & Gurley, E.S. (2014). Dynamics of Japanese encephalitis virus transmission among pigs in northwest Bangladesh and the potential impact of pig vaccination. *PLoS Neglected Tropical Diseases*, 8(9):e3166. doi:10.1371/journal.pntd.0003166

Knips, V. (2004). *Review of the livestock sector in the Mekong countries*. Food and Agriculture Organization of the United Nations. http://www.fao.org/ag/againfo/resources/en/publications/sector_reports/lsr_mekong.pdf

LaBeaud, A.D., Bashir, F. & King, C.H. (2011). Measuring the burden of arboviral diseases: the spectrum of morbidity and mortality from four prevalent infections. *Population Health Metrics*, 9:1–11. doi:10.1186/1478-7954-9-1

Lindahl, J., Boqvist, S., Ståhl, K., Thu, H.T.V. & Magnusson, U. (2012b). Reproductive performance in sows in relation to Japanese encephalitis virus seropositivity in an endemic area. *Tropical Animal Health and Production*, 44:239–245. doi:10.1007/s11250-011-0005-0

Lindahl, J., Chirico, J., Boqvist, S., Thu, H.T.V. & Magnusson, U. (2012a). Occurrence of Japanese encephalitis virus mosquito vectors in relation to urban pig holdings. *American Journal of Tropical Medicine and Hygiene*, 87(6):1076–1082. doi:10.4269/ajtmh.2012.12-0315

Lindahl, J.F., Ståhl, K., Chirico, J., Boqvist, S., Thu, H.T.V. & Magnusson, U. (2013). Circulation of Japanese encephalitis virus in pigs and mosquito vectors within Can Tho city, Vietnam. *PLoS Neglected Tropical Diseases*, 7(4):e2153. doi:10.1371/journal.pntd.0002153

Liu, W., Gibbons, R.V., Kari., K., Clemens, J.D., Nisalak, A., Marks, F. & Xu, Z.Y. (2010). Risk factors for Japanese encephalitis: a case-control study. *Epidemiology and Infection*, 138:1292–1297. doi:10.1017/S0950268810000063

Mackenzie, J.S., Williams, D.T. & Smith, D.W. (2007). Japanese encephalitis virus: the geographic distribution, incidence, and spread of a virus with a propensity to emerge in new areas. In: Tabor, E. (ed.), *Emerging Viruses in Human Populations*. Amsterdam, Netherlands: Elsevier, 201–268. doi:10.1016/S0168-7069(06)16010-3

Murty, U.S., Rao, M.S., & Arunachalam, N. (2010). The effects of climatic factors on the distribution and abundance of Japanese encephalitis vectors in Kurnool district of Andhra Pradesh, India. *Journal of Vector Borne Diseases*, 47:26–32.

Mwandawiro, C., Boots, M., Tuno, N., Suwonkerd, W., Tsuda, Y. & Takagi, M. (2000). Heterogeneity in the host preference of Japanese encephalitis vectors in Chiang Mai, northern Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 94:238–242.

OIE (2019). *Japanese encephalitis*. https://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Disease_cards/JAPANESE_ENCEPHALITIS.pdf [2019-12-17]

PATH (2010). *Japanese encephalitis vaccine introduction in Cambodia.* https://path.azureedge.net/media/documents/CP_cambodia_je_vac_intro.pdf [2020-01-20]

Peiris, J.S.M., Amerasinghe, F.P., Arunagiri, C.K., Perera, L.P., Karunaratne, S.H.P.P., Ratnayake, C.B., Kulatilaka, T.A. & Abeysighe, M.R.N. (1993). Japanese encephalitis in Sri Lanka: comparison of vector and virus ecology in different agro-climatic areas. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 87:541–548.

Poidinger, M., Hall, R.A. & Mackenzie, J.S. (1996). Molecular characterization of the Japanese encephalitis serocomplex of the flavivirus genus. *Virology*, 218(2):417–421. doi:10.1006/viro.1996.0213

Ricklin, M.E., García-Nicolás, O., Brechbühl, D., Python, S., Zumkehr, B., Nougairede, A., Charrel, R.N., Posthaus, H., Oevermann, A. & Summerfield, A. (2016a). Vector-free transmission and persistence of Japanese encephalitis virus in pigs. *Nature Communications*, 7:e10832. doi:10.1038/ncomms10832

Ricklin, M.E., García-Nicolás, O., Brechbühl, D., Python, S., Zumkehr, B., Posthaus, H., Oevermann, A. & Summerfield, A. (2016b). Japanese encephalitis virus tropism in experimentally infected pigs. *Veterinary Research*, 47:e34. doi:10.1186/s13567-016-0319-z

Rosen, L. (1986). The natural history of Japanese encephalitis virus. *Annual Review of Microbiology*, 40:395–414. doi:10.1146/annurev.mi.40.100186.002143

Ruget, A.-S., Beck, C., Gabassi, A., Trevennec, K., Lecollinet, S., Chevalier, V. & Cappelle, J. (2018). Japanese encephalitis circulation pattern in swine of northern Vietnam and consequences for swine's vaccination recommendations. *Transboundary and Emerging Diseases*, 65:1485–1492. doi:10.1111/tbed.12885

Samkol, P., Borin, K. & Sovann, S. (2008). Pig systems in Southeast Asia – the case of Cambodia. In: Thorpe, W. & Jemaneh, T. (eds.), *Pig systems in Asia and the Pacific: How can research and development enhance benefits to the poor? Proceedings of a regional workshop held in Bangkok, Thailand, 23–24 November 2006.* Nairobi, Kenya: International Livestock Research Institute, 34–42. https://hdl.handle.net/10568/1741

Scherer, W.F., Moyer, J.T. & Izumi, T. (1959). Immunologic studies of Japanese encephalitis virus in Japan: V. Maternal antibodies, antibody responses and viremia following infection of swine. *Journal of Immunology*, 83:620–626.

Schneider, R.J., Firestone, M.H., Edelman, R., Chieowanich, P. & Pornpibul, R. (1974). Clinical sequelae after Japanese encephalitis: a one-year follow-up study in Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health*, 5(4):560–568.

Schuh, A.J., Ward, M.J., Leigh Brown, A.J. & Barrett, A.D.T. (2014). Dynamics of the emergence and establishment of a newly dominant genotype of Japanese encephalitis virus throughout Asia. *Journal of Virology*, 88(8):4522–4532. doi:10.1128/JVI.02686-13

Simmonds, P., Becher, B., Bukh, J., Gould, E.A., Meyers, G., Monath, T., Muerhoff, S., Pletnev, A., Rico-Hesse, R., Smith, D.B., Stapleton, J.T., & ICTV Report Consortium (2017). ICTV virus taxonomy profile: Flaviviridae. *Journal of General Virology*, 98:2–3.

Solomon, T., Dung, N.M., Kneen, R., Gainsborough, M., Vaughn, D.W. & Khanh, V.T. (2000). Japanese encephalitis. *Journal of Neurology, Neurosurgery and Psychiatry*, 68:405–415. doi:10.1136/jnnp.68.4.405

Solomon, T., Ni, H., Beasley, D.W.C., Ekkelenkamp, M., Cardosa, M.J. & Barrett, A.D.T. (2003). Origin and evolution of Japanese encephalitis virus in Southeast Asia. *Journal of Virology*, 77(5):3091–3098. doi:10.1128/JVI.77.5.3091-3098.2003

Ström, G., Andersson Djurfeldt, A., Boqvist, S., Albihn, A., Sokerya, S., San, S., Davun, H. & Magnusson, U. (2017). Urban and peri-urban family-based pig-keeping in Cambodia: Characteristics, management and perceived benefits and constraints. *PLoS ONE*, 12(8):e0182247. doi:10.1371/journal.pone.0182247

Tarantola, A., Goutard, F., Newton, P., de Lamballerie, X., Lortholary, O., Cappelle, J. & Buchy, P. (2014). Estimating the burden of Japanese encephalitis virus and other encephalitides in countries of the Mekong region. *PLoS Neglected Tropical Diseases*, 8(1):e2533. doi:10.1371/journal.pntd.0002533

The World Bank (2019). *The World Bank in Cambodia – Overview.* https://www.worldbank.org/en/country/cambodia/overview [2020-02-09]

Thongsripong, P., Green, A., Kittayapong, P., Kapan, D., Wilcox, B. & Bennett, S. (2013). Mosquito vector diversity across habitats in central Thailand endemic for Dengue and other arthropod-borne diseases. *PLoS Neglected Tropical Diseases*, 7(10):e2507. doi:10.1371/journal.pntd.0002507

Touch, S., Hills, S., Sokhal, B., Samnang, C., Sovann, L., Khieu, V., Soeung, S.C., Toda, K., Robinson, J. & Grundy, J. (2009). Epidemiology and burden of disease from Japanese encephalitis in Cambodia: results from two years of sentinel surveillance. *Tropical Medicine and International Health*, 14(11):1365–1373. doi:10.1111/j.1365-3156.2009.02380.x

Umenai, T., Krzysko, R., Bektimirov, T.A. & Assaad, F.A. (1985). Japanese encephalitis: current worldwide status. *Bulletin of the World Health Organization*, 63(4):625–631.

WHO (2015). *Japanese encephalitis vaccines: WHO position paper – February 2015*. Geneva, Switzerland: World Health Organization. (Weekly Epidemiological Record, 90(9):69–88)

WHO (2019). *Japanese encephalitis*. https://www.who.int/news-room/fact-sheets/detail/japanese-encephalitis [2019-11-28]

Williams, D.T., Mackenzie, J.S. & Bingham, J. (2019). Flaviviruses. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. & Zhang, J. (eds.), *Diseases of Swine*. 11. ed. Hoboken, New Jersey: John Wiley & Sons, 530–543. doi:10.1002/9781119350927

Yamada, M., Nakamura, K., Yoshii, M. & Kaku, Y. (2004). Nonsuppurative encephalitis in piglets after experimental inoculation of Japanese encephalitis flavivirus isolated from pigs. *Veterinary Pathology*, 41:62–67.

APPENDICES

Appendix 1 – Blood sample form

○ Used for questions with only one possible answer

☐ Used for questions with more than one possible answer

Farm no: ………. (Example: F1)   Pig no: ………. (Example: P1)

Sex	Age	Breed	*Only sows:* Health history (last 12 months)
Female ○ Male ○	…… months *or* …… years	Indigenous ○ Exotic ○ Mix ○	Abortions ☐ Stillbirths ☐ Mummified foetuses ☐ Weakness, shaking or convulsions in piglets ☐ Number of live piglets in last litter: ………

Appendix 2 – Questionnaire

○ Used for questions with only one possible answer

☐ Used for questions with more than one possible answer

Farm no: ……….

No	***A. Household demographics***		
A1	Geographic location	Province: .... District: .... Commune: .... Village:	
A2	Sex of respondent	Female ○ Male ○	
A3	Age of respondent	.... years	
A4	Highest level of education of respondent	College/University ○ Upper secondary school ○ Lower secondary school ○ Primary school ○ No education ○	

A5	Number of people living in the household	Adults (15-60 years): ………. Children (< 15 years): ………. Elderly (> 60 years): ……….	
No	***C. Farm details***		
C1	Number of pigs (at time of visit)	Piglets (< 1 month): ………. Growers (1-3 months): ………. Fatteners (> 3 months): ………. Breeding sows: ………. Breeding boars: ……….	
C2	Pig breed(s) on the farm	Indigenous pigs ☐ Exotic pigs ☐	
C3	Housing system for the pigs	Tethered ☐ Confined in pens ☐ Partly confined ☐ Free roaming ☐	 **Go to C6**
C4	Which group(s) of pigs are tethered/confined?	Piglets (< 1 month) ☐ Growers (1-3 months) ☐ Fatteners (> 3 months) ☐ Breeding sows ☐ Breeding boars ☐	
C5	If partly confined, during what part of the day are the pigs kept confined?	Day ☐ Dusk ☐ Night ☐ Other: ………. ☐	
C6	If partly confined/free roaming, during which season do the pigs roam free?	Dry season ○ Rainy season ○ Both ○	
No	***D. Disease knowledge and vaccination***		
D15	Have you heard of Japanese encephalitis?	Yes ○ No ○	 **Go to E1**

D16	Can you explain what it is?	Yes: ………. ○ No ○	
D17	Who can get infected? *Don't read options out loud*	Humans ☐ Pigs ☐ Other animals: ………. ☐	
D18	*If answering "humans":* Do you know how people get infected?	Yes: ………. ○ No ○	
D19	Has anyone in your family or anyone else you know had Japanese encephalitis?	Yes, someone in the family ☐ Yes, someone else ☐ No ○	
D20	Are you or anyone else in the household vaccinated against Japanese encephalitis?	Yes, the whole family ○ Yes, me and/or my partner ○ Yes, the children ○ No ○	 **Go to D22**
D21	If yes, who paid for the vaccine?	The family ○ The government ○ Other: ………. ○	**Go to D23**
D22	If no, why not?	Can't afford the vaccine cost ☐ Can't get to the vaccine central ☐ Didn't know there were any available vaccines ☐ Didn't know people could get infected ☐ Afraid of vaccine side effects ☐ Other: ………. ☐	
D23	*If answering "pigs" to qn D17:* Do you know how pigs get infected?	Yes: ………. ○ No ○	
No	***E. Disease among the pigs***		
E1	Did any of the sows abort their foetuses during the past year?	Yes ○ No ○ Don't know ○	 **Go to E3**

E2	If yes, how many of the sows aborted?	Number: ………. Don't remember ○	
E3	Did any of the sows produce stillborn piglets or mummified foetuses during the past year?	Yes, stillborn piglets □ Yes, mummified foetuses □ No ○	**Go to E5**
E4	If yes, how many sows did it happen to?	Number: ………. Don't remember ○	
E5	Have any new-born piglets showed any of the following symptoms during the past year?	Weakness □ Shaking □ Convulsions □ None ○	**Go to F1**
E6	If yes, did any of the piglets die? Approximately how many?	Yes, all of them ○ Yes, half of them ○ Yes, but only a few ○ No, none ○	
No	***F. Mosquito awareness and protection***		
F1	Have you heard of diseases being transmitted to people through mosquito bites?	Yes ○ No ○	
F2	Do you use any mosquito protection in your family?	Yes ○ No ○	**Go to F4**
F3	If yes, please describe what kind.	Long-sleeved clothes □ Mosquito repellents □ Mosquito nets in windows/doors □ Mosquito bed nets □ Mosquito coils □ Electric rackets □ Lids on water tanks □ Other: ………. □	**Go to F5**

F4	If no, why not?	Can’t afford the products ☐ Takes too much effort ☐ Didn’t know you had to protect yourself against mosquito bites ☐ Other: ………. ☐	
F5	Do you protect your pigs against mosquitos in any way?	Yes ○ No ○	 **Go to F7**
F6	If Yes, how?	Use mosquito nets ☐ Use insect repellents ☐ Minimize water sources near the pigs ☐ Other: .... ☐	
F7	*Observational question:* Is any of the following within sight of the grounds?	Rice field ☐ Swamp ☐ Lake ☐ Pond ☐ Well ☐ Water tank ☐ Other stagnant water: ………. ☐	