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**Faculty of Veterinary Medicine
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Alternate wetting and drying and its influence on zoonotic disease vectors in northern Vietnam

Can changing the rice-growing method reduce the presence of Japanese encephalitis virus?

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SUMMARY

Japanese encephalitis is an important disease affecting 70,000 people every year, with young children constituting the major part. Growing rice with alternate wetting and drying (AWD) methodology reduces water consumption at the same time as it contributes to a reduction in greenhouse gas emission. In this study, mosquitoes and mosquito larvae were collected at rice-growing farmers' households, comparing the number of mosquitoes at households using AWD with households practicing conventional methods. Mosquitoes were also analyzed with PCR regarding Japanese encephalitis virus. In addition, a survey was conducted at the households, concerning the farmers knowledge about mosquito-borne diseases in general, Japanese encephalitis in particular and their measurements taken to avoid these diseases. In total, 2,072 mosquitoes were trapped, identified and analyzed. No difference in the number of mosquitoes were found between AWD-practicing households and households practicing conventional rice growing. None of the mosquito pools tested positive for Japanese encephalitis virus. Correlations between level of education and knowledge about mosquito-borne diseases were found. Knowledge about these diseases needs to be improved to increase the preventions practiced. This study shows that more research, with a greater number of material and in areas with different constitutions between AWD and conventional rice-growing methods, is needed.

CONTENT

INTRODUCTION

Annually, the human global incidence of Japanese encephalitis (JE) is approximately 70,000 (Campbell *et al*., 2011). About 75% of these cases occur in children 0-14 years old (Campbell *et al*., 2011). JE is associated with irrigated rice agriculture and is largely a disease of rural areas (Solomon, 2000; van den Hurk *et al*., 2009). The number of people living in rural areas where JE is endemic or epidemic is calculated to 1.9 billion people (Keiser *et al*., 2005). Studies that show that JE is not only a rural disease makes the number of people at risk for transmission of the zoonotic disease markedly higher (Lindahl *et al*., 2013). JE is present in south and southeast Asia and northern Australia. In Asia it is the main cause of viral encephalitis. The transmission between animals is carried out by mosquitoes (Solomon, 2000). Flooded rice fields are the most important breeding habitat for the vector mosquitos (van der Hoek *et al*., 2001). Pigs are also important in the transmission since they are amplifying the virus and live in close relation to humans (van den Hurk *et al*., 2009).

Vietnam is an elongated country in southeast Asia. In 2017 the number of inhabitants were just over 95.5 million. 64% of the inhabitants are living in rural areas (General Statistics Office of Viet Nam, 2019). Nearly half of the labour works in farming. Rice is the dominating crop and Vietnam is one of the biggest exporters of rice in the world. Many Vietnamese breed pigs, either for subsistence or selling. (Utrikespolitiska Institutet, 2019). In 2018 the country had 28.2 million pigs (General Statistics Office of Viet Nam, 2019). JE is the leading cause of viral CNS infection in Vietnam, followed by dengue virus, herpes simplex virus and enteroviruses. High mortality and morbidity is associated to these diseases. (Tan *et al*., 2014).

Alternate wetting and drying (AWD) is a newly developed water-saving technique when growing rice. Instead of the rice field being continuously flooded, it gets intermittently dry and aerated. This reduces not only the water consumption but also the emission of greenhouse gas (Sander *et al*., 2016; van der Hoek *et al*., 2001).

The overall objective with the study was to investigate if the AWD technique has benefits in form of reduced presence of the total number of mosquitos and thus less JEV carrying mosquitoes. The research question is highly relevant and AWD has the potential to both contribute to public health benefits concurrently with climate change mitigation. By assessing and highlighting potential public health benefits of the AWD technique it can be promoted and encouraged in wider areas.

LITERATURE REVIEW

Japanese encephalitis

The virus

Japanese encephalitis virus (JEV) is a single-stranded RNA virus (Solomon, 2000). It belongs to the family *Flaviviridae*. The family *Flaviviridae* consists of three genera; pestiviruses, hepaciviruses and flaviviruses. JEV belongs to the latter. Flaviviruses are also called arthropod-borne viruses since they are mostly transmitted to humans by mosquitos or ticks, dividing them in subgroups. The Mammalian tick-borne group are all tick-borne viruses. The Dengue virus group, Yellow Fever virus group and Japanese encephalitis group belongs to the mosquito-borne viruses. Further, the Japanese encephalitis group consists of West Nile encephalitis, St Louis encephalitis, Murray Valley encephalitis and Japanese encephalitis. (Unni *et al*., 2011). All the viruses in this serological group cause significant mortality (van den Hurk *et al*., 2009). West Nile virus is present in parts of Europe, the Middle East and Africa. Murray Valley encephalitis and St Louis encephalitis are present in Australia and North America respectively. (Solomon, 2000). Currently, JEV is divided into five genotypes, I-V (Mohammed *et al.*, 2011; Uchil & Satchidanandam, 2001). Genotype I-III are the most widespread and similar to each other (Pan *et al*., 2011; Solomon *et al*., 2003).

Epidemiology

The natural transmission cycle of JEV is between mosquitoes, ardeid birds and pigs (Solomon, 2000). Pigs and birds are amplifying hosts. By feeding on viraemic pigs or birds, mosquitoes get infected, spreading the virus further. (Solomon, 2000; van den Hurk *et al*., 2009). These natural hosts do not develop encephalitis (Solomon, 2000). Humans as well as other animals, such as dogs, horses and cattle, are not considered to be a part of the natural transmission cycle. They do not develop viremias high enough to infect a novel mosquito. Therefore, they are considered dead-end hosts. They are incidentally infected when staying close to the natural transmission cycle. (Gould *et al*., 1974; Solomon, 2000). Humans and horses are dead-end hosts that can develop clinical disease and sometimes fatal encephalitis (van den Hurk *et al*., 2009). When the number of mosquitoes increase, the number of infected mosquitoes and the infection rate of pigs increases as well. Soon after that, there is an increase in human infection too (Solomon, 2000). Even though they do not develop a high viremia, cattle, dogs, goats and rodents have shown seroconversion (van den Hurk *et al*., 2009). Dogs can thus be useful as sentinels for human infection since they often live in close relations (Shimoda *et al*., 2010).

The primary enzootic hosts of JEV are considered ardeid wading birds. They are one explanation to how the virus can spread to new areas. Pigs, on the other hand, are considered the most important amplifying host since they are kept in close relationship to humans. Their viremia is high enough to infect mosquitoes for up to four days and they have a high birth rate which serves a high rate of new susceptible pigs (Solomon, 2000; van den Hurk *et al*., 2009). Normally, JEV does not cause any disease in pigs however under certain conditions, it can cause abortions, stillbirths, mummified foetuses or weak-borne piglets. This if the sow or gilt is infected before 60-70 days into pregnancy (Daniel Givens & Marley, 2008).

In tropical areas, the epidemical pattern of JEV is endemic while it is epidemic in subtropical and temperate regions. Most commonly, the epidemic outbreaks in subtropical and temperate areas occur after the rainy season, in summer and early autumn (Solomon, 2000; van den Hurk *et al*., 2009). In tropical endemic areas, like in the southern Vietnam, almost all pigs get infected before sexual maturity. Northern Vietnam has a subtropical environment, in consequence, JE is epidemic. Between July and September only 80% of the pigs in northern Vietnam will be protected against JE before they reach sexual maturity, hence the risk of reproductive disorders is higher (Ruget *et al*., 2018).

A study in Japan have shown that JEV seems to be surviving locally between seasons in epidemic regions as well as being reintroduced (Nabeshima *et al*., 2009). Recent studies have also shown that through direct contact, transmission between pigs can occur (Diallo *et al*., 2018; Ricklin *et al*., 2016). The transmission occurs through oronasal secretions and pigs have shown to be highly susceptible to oronasal infection. Consequently, JEV can be transmitted without arthropod vectors. Adding this to the epidemiological cycle can have high impact in temperate areas, such as northern Vietnam (Ricklin *et al*., 2016).

Vectors

In total, JEV has been isolated from over 30 different mosquito species (van den Hurk *et al*., 2009). Among these are several *Aedes* species, (e.g. *Aedes. albopictus*) and a few species of *Anopheles, Mansonia and Armigeres* (Rosen, 1986). The main vectors of JEV, however, belongs to the *Culex* genus (Gould *et al*., 1974; Oliveira *et al*., 2017).

The primary, as well as most important, mosquito for infection of JEV is considered to be *Culex tritaeniorhynchus* (Rosen, 1986; Solomon, 2000; van den Hurk *et al*., 2009). It belongs to the *Cx. vishnui* subgroup along with *Cx. vishnui* and *Cx. pseudovishnui* which has yielded several isolates as well (Gould *et al*., 1974). Additionally, other *Culex* spp. species such as *Cx. gelidus*, *Cx. fuscocephala* and *Cx. annulirostris* have yielded numerous isolates. They are considered important as secondary vectors or as regional vectors and have all been proven to be efficient vectors in the laboratory, along with the species of the *Cx. vishnui* subgroup. (Unni *et al*., 2011; van den Hurk *et al*., 2009). In northern Australia, where *Cx. tritaeniorhynchus* and *Cx. vishnui* do not occur, *Culex annulirostris* is the reported vector for JEV (Hurk *et al*., 2003; van den Hurk *et al*., 2019). Two other *Culex* species, *Cx. orientalis* and *Cx. pipiens* have been tested positive for JE genotype V in Korea in 2012 (Kim *et al*., 2015).

In southeast Asia, there are 42 species of *Culex* spp. present (Sirivanakarn, 1976). Common *Culex* spp. vectors in Vietnam are *Cx. alienus, Cx. bitaeniorhynchus, Cx. fuscocephala, Cx. gelidus, Cx. infula, Cx. pseudovishnui, Cx. quinquefasciatus, Cx. sitiens, Cx. tritaeniorhynchus, Cx. vishnui, Cx. whitei* and *Cx. whitmorei* (Reuben *et al*., 1994). Multiple genotype I isolates of JEV are circulating countrywide and has been isolated from mosquitos belonging to the *Cx. tritaeniorhynchus* and *Cx. vishnui* family; *Cx. tritaeniorhynchus* in northern and southern parts of the country and from *Cx. vishnui* in the highlands. (Kuwata *et al*., 2013).

The extrinsic incubation period in the mosquito, before it can transmit the virus, further varies with temperature from 6-20 days. Infection rates varies with many aspects. Climate, agriculture practises and presence of amplifying hosts are a few examples. (van den Hurk *et al.*, 2009).

Seasonal pattern have been linked to the onset of the monsoon season (Gajanana *et al*., 1997). Besides, different genotypes of JEV can have different infection rates. As an example, genotype III and Ib have a higher infection rate than genotype Ia in the *Cx. quinquefasciatus* mosquito. (Huang *et al*., 2016).

Feeding preferences

Overall, cattle and pigs followed by cats and dogs are in the lead when it comes to feeding preferences for vector mosquitoes. When it comes down to mosquito species level, the feeding preferences deviates. (Oliveira *et al*., 2018). Most of the vectors for JEV are opportunistic feeders, but pigs are the main blood feed for *Cx. vishnui* and cattle is the main blood feed for *Cx. tritaeniorhynchus*, *Cx. gelidus* and *Cx. fuscocephalus*. Host feeding preferences are therefore another consideration in the epidemiology of JE. (Gould *et al*., 1974; Mitchell *et al*., 1973).

After cattle, which is the major feeding host for *Cx. tritaeniorhynchus*, pigs come second, accounting for only a small part. Goats, dogs and chicken accounts to an even smaller part, and the feeding on humans further less (Gould *et al*., 1974; Liu *et al*., 2011). Human feeding has been accounted to be as low as 1.5% and *Cx. tritaeniorhynchus* has been described as a highly zoofagic species (Samuel *et al*., 2008). The feeding behaviour is thought to be of physiological conditions instead of inherited behaviour and the availability of different host plays a part in the feeding pattern (Mwandawiro *et al*., 2000).

There are other species of mosquitoes that have been shown to feed on pigs to an equal proportion as *Cx. tritaenorhynchus* but to a much higher proportion on humans. In India, *Ma. uniformis* have been seen to feed on humans to 12.5%, considered an important secondary regional vector for JEV (Samuel *et al*., 2008).

Risk factors

Living close to rice fields as well as having pigs in the family or neighbours with pigs are known risk factors for JE (Liu *et al*., 2010). The proportion of rice field land is positively correlated to the presence of *Cx. tritaeniorhynchus* (Richards *et al*., 2010). The rice production in areas where JE is endemic has increased to a high degree during the last decades, along with the areas used for the production (Keiser *et al*., 2005).

Many environmental factors have been investigated as risk factors for JE. Factors such as average monthly temperature, relative humidity, and precipitation are positively correlated to the incidence. (Bi *et al*., 2007; Lee *et al*., 2017). Monthly mean air pressure has on the contrast seen to be inversely correlated (Bi *et al*., 2007). Regarding relative humidity, the rise in incidence is dramatic when it rises above 80%. A 100 mm rise in precipitation in the preceding and same month has been found to correlate with about a 20% increase in viral encephalitis (VE) incidence. Further regarding precipitation, the incidence of VE increases when the average precipitation rises to its mean value of 130 mm. When the precipitation rises above that, the incidence gradually declines (Lee *et al*., 2017). How the weather variabilities influence the transmission of JE is of authors speculated to be complicated. It is thought to affect the breeding conditions, where high temperature, humidity and precipitation is favourable. (Bi *et al*., 2007; Lee *et al*., 2017; Solomon *et al*., 2003). High temperature is favourable in the way that it shortens the extrinsic incubation period in the mosquito and abbreviates the time it takes for larvae to develop (Bi *et al*., 2007; van den Hurk *et al*., 2009). Humidity has a positive influence in the longevity of mosquitoes and in that they can disperse farther. Rainfall provides more breeding habitats for the mosquitoes (Bi *et al*., 2007). Typhoons or high rainfall may in contrast reduce the mosquito population by washing away the larvae (Lee *et al*., 2017). Furthermore, weather variabilities can have different influences in different regions *(Bi et al., 2007; Lee et al*., 2017). Weather variabilities may also affect human behaviour. As an example, higher temperature can increase peoples exposure time to mosquitoes due to practising more activities outdoor (Bi *et al*., 2007). Socioeconomic status is another factor that remains to be investigated as a risk of JE (Bi *et al*., 2007; Lee *et al*., 2017).

Preventions

Extensive use of pesticides in rice-growing areas have been seen associated with lower densities of adult mosquitoes (Self *et al*., 1973). Other biological and environmental vector control managements have also been evaluated, finding that the use of irrigated rice agriculture integrated with the use of larval eating fish can reduce vector populations in the rice field (Keiser *et al*., 2005). Another prevention method that has been suggested is that an increased availability of cows might be useful as an effective control of JE, since the mosquitoes would be drawn to the cows rather than the pigs (Mwandawiro *et al*., 2000).

Vaccination of future breeding pigs have been suggested useful in epidemic areas such as northern Vietnam where not all pigs are accounted to have been naturally infected before sexual maturity. This to avoid the occurrence of JE-associated reproductive disorders (Ruget *et al*., 2018).

Concerning humans, vaccination should be the prioritized control measure of the disease since it is the prevention that gives a long-term sustainable protection (Yun & Lee, 2014). The World Health Organization (WHO) recommends schedules for immunization in all areas having JE as a public health issue. Four different types of vaccines are in use where a live attenuated is the one used most commonly. (World Health Organization, 2019).

JE in humans

The number of people developing clinical features from the infection of JE varies from 1 in 25 to 1 in 1000. It is most commonly a disease of children and young adults. The case fatality of hospitalized JE patients is about 30%. (Solomon, 2000; van den Hurk *et al*., 2009). Most commonly, infection in humans does not show any clinical signs, or results in an illness that looks like the flu, which is not specific for JE. The spectrum of clinical manifestations is broad and there are many atypical cases. More typical clinical signs are starting with a few days of fever, followed by headache, vomiting and a reduced level of consciousness. The fever is non-specific and may include diarrhoea, coryza and rigidity. Atypical cases are for example cases of older children and adults showing abnormal behaviour as the only clinical sign. There is no specific cure for the disease. Supportive treatment includes restraining of the intracranial pressure and controlling the convulsions. Neurological sequelae follow half of the patients surviving the disease (Solomon, 2000).

JE in Vietnam

Both genotype III and I are circulating in Vietnam (Kuwata *et al*., 2013; Lindahl *et al*., 2013; Solomon *et al*., 2003). Genotype 1 is currently the dominating strain in pigs, birds and humans and is by recent studies thought to replace genotype 3 in Vietnam as well as in other parts of Asia (Do *et al*., 2015).

Yen *et al*. (2010) reports that the annual human incidence of acute encephalitis syndrome (AES) in Vietnam varies from 1.4-3.0 people per 100 000 inhabitants and that there has been a decreasing trend during the ten-year period between 1998 and 2007. The total number of AES cases from 1998-2007 was 19,354 and the number of deaths due to AES accounted to 741. In the northern region of the country, the mean annual incidence was highest. The lowest incidence was seen in the highlands. The case fatality was consistently 3.8% of all AES cases during the whole ten-year period. A peak in AES cases were seen during the summer months, but this pattern was primary seen in the north. Seventy three percent of the AES cases in the northern region occurred from May through September. During 2004 and 2005, AES cases in five northern provinces were further analysed laboratory regarding JE. Of 421 AES cases tested, 52% were positive regarding recent JEV infection (Yen *et al*., 2010).

Diagnostics

In humans, virus isolation is very difficult due to the low level of viremia and the rapid production of antibodies that neutralizes the virus. The most practical way to diagnose is serologically by enzyme linked immunosorbent assay (ELISA) (Solomon, 2000). Due to a lack of diagnostic facilities, human viral encephalitis (VE) cases are often reported and considered as a representative for JE surveillance since it is considered the leading cause of VE. Enteroviruses and Dengue-virus is the second most important. (Solomon *et al*., 2002; Tan *et al*., 2014; Yen *et al*., 2010).

Detection of JEV in mosquitoes

Traditionally, to analyse mosquitoes for JEV they are trapped, identified to species and analysed in pools. Then there are different methods to detect the virus. Virus inoculation in vivo (e.g. mice) has to a large scale been replaced by inoculation in vitro (e.g. arthropod cells) but is still gold-standard. Further, detection of viral RNA by real-time polymerase chain reaction (PCR) protocols is an enhancing technique that is more rapid, sensitive and specific. Additionally, discovery of unidentified isolates and genetic characterisation is determined by sequencing technologies that analyses the viral nucleic acid (Hall *et al*., 2012).

In mosquitoes, viral RNA can be stable for up to 14 days post-mortem in hot summer field conditions. Viable virus can usually no longer be detected after 1-day post-mortem. The pooling of mosquitoes is for practical and economic reasons. Still, when pool size increases the ability to find virus decreases. A single infected mosquito has been detected in pools with 200 or less mosquitos using an RT-PCR (Johansen *et al*., 2002). Improved sensitivity for detection of JEV has been seen when samples were diluted and with the use of a nested/semi-nested PCR (Johansen *et al*., 2002; Lindahl, 2012).

In a study of Liu *et al.* (2012) a more rapid method of JEV detection than conventional RT-PCR and real-time RT-PCR, was developed. The method is based on reverse transcription loop-mediated isothermal amplification (RT-LAMP). The sensitivity is similar to real-time RT-PCR but ten times higher than conventional RT-PCR. It did not show any cross-reactivity to other flaviviruses, making the specificity high. What makes the method more rapid is that the amplification step can be completed in a single tube within 50 minutes. The method does not need expensive equipment such as thermal cyclers (Liu *et al*., 2012).

Minimum infection rate (MIR) is defined as the ratio of the number of positive pools to the total number of mosquitoes in the sample. It is appropriate to use to calculate the estimated infection rate when the mosquito population is at a low level of infection, since it assumes that there is only one infected mosquito in each positive pool. Maximum likelihood estimation (MLE) is another, more complicated, algorithm to calculate the estimated infection rate. It considers variations in pool size and is more suitable when the infection levels are high (Bustamante & Lord, 2010).

Alternate wetting and drying

Alternate wetting and drying (AWD) is a water-saving method in rice growing. The method is based on intermittent drying of the rice fields compared with conventional rice growing where the field is continuously flooded (van der Hoek *et al*., 2001). Furthermore, it aerates the rice field and therefore reduces the emission of the greenhouse gas (GHG) methane, which conventional flooded rice fields is a large source of (Sander *et al*., 2016). Rice production stands for 1.2% of all GHG emissions globally. In a rice-growing country it is higher, for example 13% of all GHG emissions in the Philippines (Sander *et al*., 2017). AWD can reduce the methane gas emission from the rice field with up to 43%. In addition, less use of irrigation water in many cases leads to less pumping of water which contributes to less use of diesel for the pumps. If the method is practised correctly, it does not cause any yield decline. It rather causes a yield increase, and reduces irrigation water consumption with 15-30% (Sander *et al*., 2016). In a previous study by Keiser *et al.* (2005) analysing the effect of AWD on densities of JE vectors showed a reduction by 14–91% in rice fields applying AWD. The effect this method may have on the incidence of JE however remained to be investigated when this article was published in 2005 and has remained since then. (Keiser *et al*., 2005).

The incentive for the farmer is what implementation of AWD strongly depends on. In the Philippines it has been seen that the incentive is directly linked to the irrigation system. The technology has been easily adopted when farmers could achieve direct financial saving due to reduced diesel use. Farmers have been found to be unwilling to implement water-saving techniques where they pay seasonal fees independent of water use (Sander *et al*., 2016).

The technique

The term AWD is synonymous with several terms, which all are alternatives to the continuous flooding, such as controlled or intermitted irrigation as well as multiple aerations. The intervals of non-flooded conditions depend on soil type and weather and varies from one day up to ten days or even more (Sander *et al*., 2016). In the AWD technique farmers are taught to use a perforated water tube for measuring the water depth in the rice field. The field is drained until the water level reaches 15 cm below the soil surface. After this, the field is re-flooded to a depth of five cm. Apart from the flowering stage, this irrigation scheme is done throughout the crop season. This is called “safe AWD” because of the roots of the rice plants still being able to capture water when the depth of the water is 15 cm below soil surface (Lampayan *et al*., 2009).

MATERIALS AND METHODS

Study areas

The two different provinces chosen for the study, Hai Duong and Thai Binh, are neighbouring provinces located east and southeast of Hanoi. Hai Duong has an area of 1,668.2 km^2. The number of inhabitants were 1,807.5 million in 2018. Thai Binh has an area of 1586,4 km^2 and a population of 1,793.2 million inhabitants (2018). This makes the population densities similar, about 1,100 inhabitants per km^2. (General Statistics Office of Viet Nam, 2019). All field work in Hai Duong took place in the village An Duong located in the An Lam commune in the Nam Sach district. The field work in Thai Binh took place in the villages Quang Trung and Le Loi, both located in the Dong Xuan commune belonging to the Dong Hung district.

Mosquito and larvae collection

Mosquitoes and larvae were collected during three field trips to each province in 2019. The first trip in the beginning of August, the second in the beginning of September and the third in the beginning of October. During each field trip at each province, addresses of 40 different households were provided by the local cooperative centre in the villages. Of these 40 households, 20 households were practicing AWD and 20 households were practicing conventional methods. The local cooperative centre also identified which rice field plot that belonged to each household. Coordinates for the households and rice field plots were obtained using smartphones with the Google Maps® application.

During the first and second field trip, larvae were searched for in the water at each household’s rice field plot and in stagnant waters at each household. During the third fieldtrip, larvae were searched for at each household but not in their field due to the rice being harvested. The larvae were searched for and collected using small plastic containers and pipettes, then stored in small glass cans in cooling boxes and fridges before transport to the laboratory.

Mosquitos were collected at each household during each fieldtrip. Since *Cx. tritaeniorhynchus* is a specially phototactic mosquito (Reisen *et al*., 1976), mosquitoes were collected using Centre of Disease Control (CDC) mini light traps. A fan, which is situated under the lightbulb, creates vacuum which draws the mosquitoes into a net-bag placed under the fan (Lindahl, 2012). Two traps were set up at each household. One trap was positioned near the sleeping place of the household owners and the other trap positioned outside, close to the livestock if there were any. Since *Culex* species most often feed during night (Gould *et al*., 1974), the traps were turned on in the evening, before dusk, stayed on during the night following collection of the trapped mosquitoes in the early morning, after dawn. Then the mosquitoes were killed by freezing and stored in tubes before being sent to the laboratory. There they were stored in -70°C until identification.

After being trained in the morphological identification process and identifying a part of the sample, the greater part of the identification process was conducted by the entomology and zoology department at the National Institute of Hygiene and Epidemiology (NIHE) in Hanoi. Mosquitoes were identified according to an illustrated key by Stojanovich *et al.* (1966). *Culex* spp. and *Aedes* spp. female mosquitoes were identified down to species. Attempts to separate species in the *Cx. vishnui* subgroup were not made due to them being difficult to identify as adults. Characteristics can overlap between these species as well as being variable within the species (Reuben *et al*., 1994). Molecular identification using PCR assays are a better way to identify these species (Toma *et al*., 2000), but was not done in this study. Remaining females and the male mosquitoes were identified to genus. Larvae were identified to genus, sorted as *Culex* spp., *Aedes* spp. or others/unidentified. No attempts were made to separate male and female larvae. After identification, the mosquitoes were stored in tubes with absolute ethanol (>99.7%) at -70°C.

PCR-analysis

PCR-analyses were started by me and the team members at the virology department at the National Institute of Veterinary Research (NIVR) in Hanoi. The remaining analyses was conducted by the virology department. The mosquitoes were pooled according to species, sex and province. Some different species of the same sex and from different provinces were pooled together to get an adequate amount of test material in that pool. There were at maximum 50 mosquitoes in each pool. After homogenisation, 500 µl of minimal essential medium (MEM) (Gibco 61100-103) were added to each pool prior to centrifugation for five minutes at 14,000 rpm at 4°C. 50 µl of the sample homogenate were then transferred into microcentrifuge tubes (RNAse free) and stored in -70°C until RNA extraction following day.

RNA extraction

The RNA extraction procedure was performed using a Qiagen RNeasy® Mini Kit. For lysis of the samples, 350 µl of Buffer RLT (added with 2-mercaptoethanol ratio 1:100) were added to each sample tube. Tubes were then vortexed for 15 seconds and spun down before adding 350 µl of 70% ethanol (diluted from absolute ethanol 96-100% with RNAse free distilled water). Further, samples were vortexed again for 15 seconds prior to centrifugation for five minutes at 5,000 rpm. Four hundred µl of lysed sample were then transferred to a spin column with a collection tube, centrifuged for one minute at 10,000 rpm, repeated twice with the content in the collection tube being discarded in between. Washings of the samples were then performed. For the first washing, 700 µl of RW1 buffer were added to each spin column, followed by centrifugation for one minute at 10,000 rpm before being placed in a new clean collection tube. For the second washing, 500 µl of RPE buffer (added with 220 ml ethanol 96-100%) were added to each spin column, again centrifuged for one minute at 10,000 rpm. The content in the collection tube was then discarded and another 500 µl of RPE buffer were added to each spin column before being centrifuged for two minutes at 14,000 rpm. Again, the content in the collection tube was discarded and samples centrifuged for one minute at 14,000 rpm. For elution of the RNA, each spin column was placed in a 1,5 ml tube and 50 µl of RNAse free distilled water were added. The columns were then incubated at room temperature for three minutes before being centrifuged for one min at 10,000 rpm. The tubes containing the RNA solution were then stored at -22°C for two hours until PCR. All the centrifugations were performed at room temperature.

Quantitative real time reverse transcription-PCR

The Qiagen QuantiTect SYBR® Green RT-PCR Kit (1000rxn) were used for the rRT-qPCR. Samples were analysed regarding flaviviruses using the 2 forward primers Flavi all S (5´-TACAACATGATGGGGAARAGAGARAA-3´) and Flavi all S2 (5´-TACAACAT-GATGGGMAAACGYGARAA-3´) together with the reverse primer Flavi all AS4 (5´-GTGTCCCAGCCNGCKGTRTCRTC-3´) (final concentration of each primer 0.4 µM). One negative control and three positive controls; dengue 1, dengue 2 and JEV were used. 20 µl of Master Mix were prepared for each RNA templates containing 4.25 µl nuclease free water, 12,5 µl QuantiTect master mix, 1 µl of each primer and 0.25 µl QuantiTect RT mix. Five µl of each RNA template were added to the 20 µl Master Mix making the total sample volume 25 µl.

The reverse transcription and amplification were run in a BIO-RAD iQ5 multicolor real-time PCR detection system. Reverse transcription was accomplished in 30 min at 50°C followed by an initial activation for 15 min at 95°C. Further, PCR was carried out by 15 seconds of denaturation at 94°C, 30 seconds of annealing at 60°C and 30 seconds of extension at 72°C, repeated for 45 cycles. Lastly, melting curves were calculated at 60-95°C for ten seconds. Results shown in BIO-RAD iQ5 2.0 software.

Questionnaire

A survey was conducted at each household during the second and third field trips. The questionnaire included questions about farmers knowledge about mosquito-borne diseases in general, JEV in particular, measurements taken to avoid mosquito bites and their perception of the number of mosquitos in the area. A score was created regarding the knowledge items measured. This knowledge score included listing diseases transmitted from mosquitoes, listing breeding sites of mosquitoes, listing risk factors for getting mosquito-borne diseases, listing symptoms you can get from mosquito-borne diseases and listing ways to prevent oneself from getting mosquito-borne diseases.

Descriptive statistics were used to evaluate most of the results. In addition, Chi2-test, t-test and regression analyses (logistic and linear) were performed in the STATA 14.2 software. To get a normal distribution, mosquito values were created using log(number of mosquitoes +0.5).

RESULT

Mosquitoes, larvae and PCR

The total number of mosquitoes caught was 2,072. Of these 1,934 were females and 138 males. 1,574 from traps outside and 498 from traps inside. The total mosquitoes caught at one household ranged from 0-232 mosquitoes with an average of 13 and median of six. The most common species found was *Cx. tritaeniorhynchus*, 1,050 mosquitoes. There was no significant difference in the total number of mosquitoes between Hai Duong and Thai Binh. Regarding the total number of *Cx. tritaeniorhynchus*, Hai Duong had a significant higher number (p= 0.0001). There was no significant difference in the total number of mosquitoes between households practicing AWD and household practicing conventional methods. There was also no difference in the total number of *Cx. tritaeniorhynchus* between the two methods. For the PCR, mosquitoes were divided into 75 different pools which all tested negative.

Larvae were found at 41 different households. The total number caught was 1,725 larvae. Of these, 711 in Hai Duong and 1,014 in Thai Binh. From households practising AWD, 1,030 larvae and from households practising conventional methods, 695 larvae. The number of larvae found at one household ranged from 0-208. No larvae were found in the rice fields.

For each person in the household, the total number of mosquitoes increase with 10^0.1 (1.3 mosquitoes (p=0.053)), mainly due to an increase of *Cx. quinquefasciatus* (p=0.054). At time of the survey, a total of seven households kept pigs, 116 households kept one or more dogs and one household kept cows. The household that kept cows did not keep any pigs. For every pig in the household the number of *Cx. tritaenorhynchus* mosquitoes increased with 10^0.2 (1,6) mosquitoes (p=0.039). For every cow the number of total mosquitoes was predicted to increase with 10^1.8 (63) mosquitoes (p=0.003) but the number of *Cx. tritaeniorhynchus* with 10^2.18 (151) mosquitoes (p=0.001). Every dog resulted in an increase of 10^0.15 (1.4) *Cx. tritaeniorhynchus*, but this was not significant (p=0.09).

Questionnaire

General information

A total number of 160 rice-growing households answered the questionnaire, 80 in each province. Of these, 63% were men and 37% women, with an average age of 59 years. The age ranged from 31-82 years. The majority, 59%, had secondary school as their highest level of education. A small part, 6%, did not have any education, 22% had high school education and 2% had university/college education. Seventy two percent had farming as their main occupation. The number of people in the household ranged from one to eleven with an average as well as median of four persons (n=159). The number of children under 15 years old in the household ranged from zero to six with an average and median of one child (n=139). Ninety six percent of the households have tap water as a source of water use, 37% use rain water, 19% use a well and 3% use a river/lake nearby (more than one option could be chosen, n=158). Family income per month ranged from 405,000 Vietnamese Dong (VND) – 70,000,000 VND with the median being 9,500,000 VND (n=142). Average total area for rice production was 2,150 m^2 with a range from 360 m^2 – 21,600 m^2 (n=158).

Awareness about mosquito-borne diseases in general and JEV in particular

Regarding which time of the day interviewed farmers said that they see the most mosquitoes, 86% answered during the evening (n=157). When we asked during which season they find the most mosquitoes, 67% answered spring and 20% answered summer (n=158), both belonging to the rainy season. Result presented in figure 1.

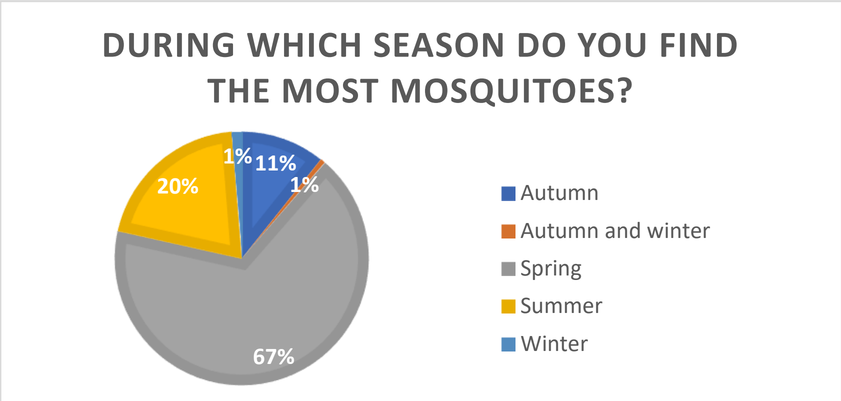

Figure 1. *Seasons where interviewed find the most mosquitoes.*

Eighty two percent of the interviewed farmers had heard about diseases being transmitted from mosquitoes to humans, presented in figure 2 (n=153). Comparing that result with the highest level of education gave a correlation presented in figure 4. The higher level of education, the higher proportion of the interviewed farmers had heard about diseases being transmitted from mosquitoes to humans. All the interviewed with college/university education (four persons) had heard about the fact.

Regarding the question if the interviewed had heard about diseases being transmitted from mosquitoes to pigs, 25% of the interviewed had heard about the fact, result presented in figure 3 (n=155). No correlation with the level of education were found in this question.

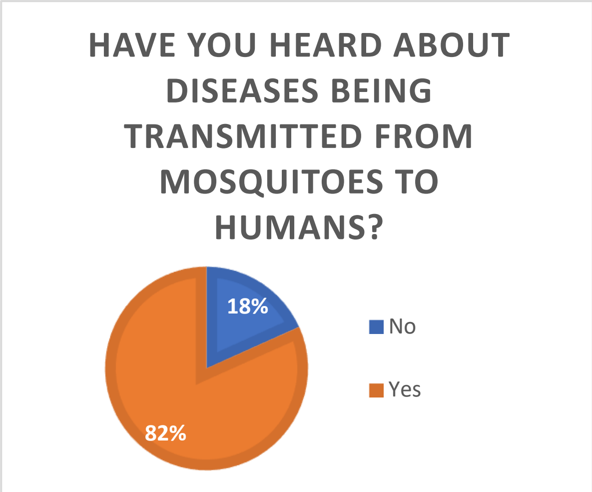

Figure 2. *Interviewed farmers' awareness of diseases being transmitted from mosquitoes to humans.*

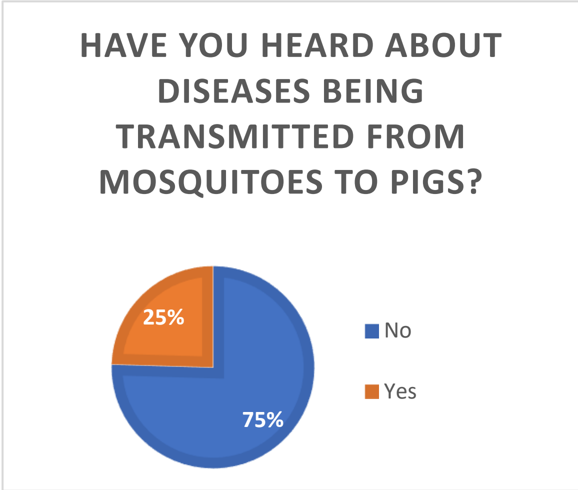

Figure 3. *Interviewed farmers' awareness of diseases being transmitted from mosquitoes to pigs.*

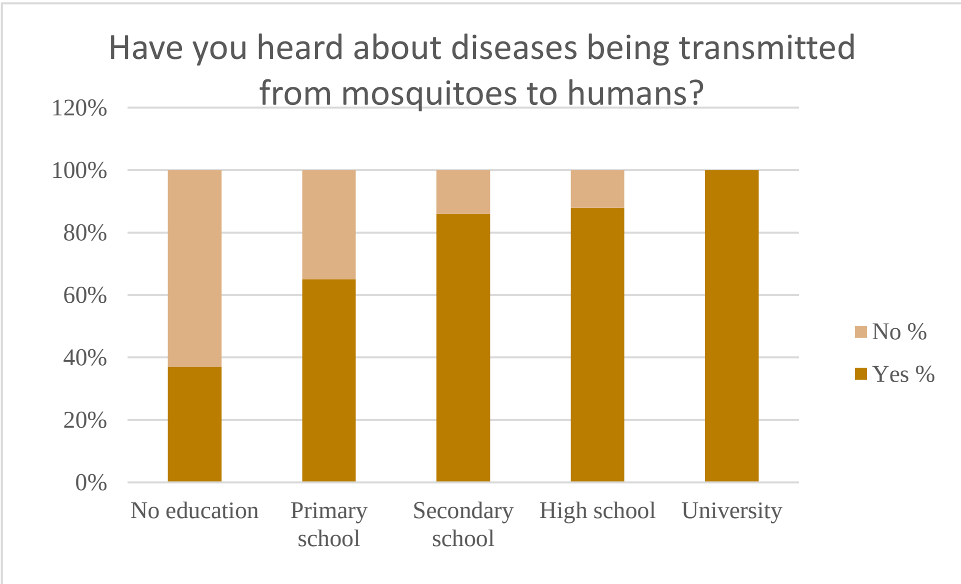

Figure 4. *Correlation between highest level of education and awareness of diseases being transmitted from mosquitoes to humans.*

Of the mosquito transmitted diseases; dengue fever, JE, zika virus disease, malaria and filariasis, dengue fever was the disease that the most interviewed, 86%, could list (n=154). Fifty five percent could list malaria, 49% JE and 5% zika virus disease. None mentioned filariasis and 5% could list other diseases. More than one disease could be listed. Result presented in figure 5. Thirty nine percent could list one, 17% could list two, 38% could list three, 3% could list four and 3% could not list any disease.

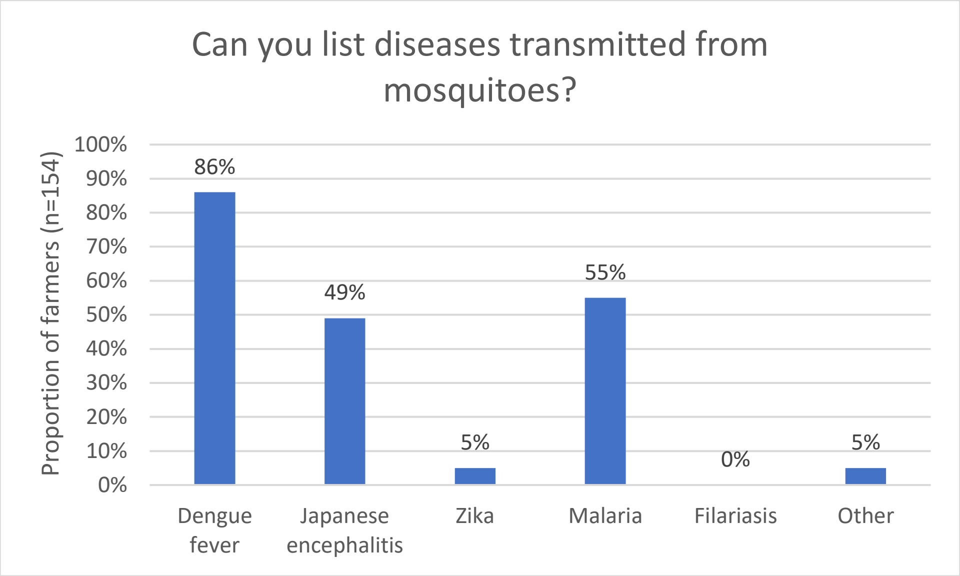

Figure 5. *Proportion of farmers that could list diseases transmitted from mosquitoes. Multiple answers were possible.*

When asked to list breeding sites of mosquitoes, stagnant water containers, drains/polluted water and water tanks were the breeding sites most known. Forty-nine households (31%) knew that rice fields are a breeding site of mosquitoes (n=157). More than one breeding site could be listed. Result presented in figure 6. Thirty five percent could list one, 15% could list two, 11% could list three, 3% could list four, 30% could list five and 1% (one household) could list six breeding sites.

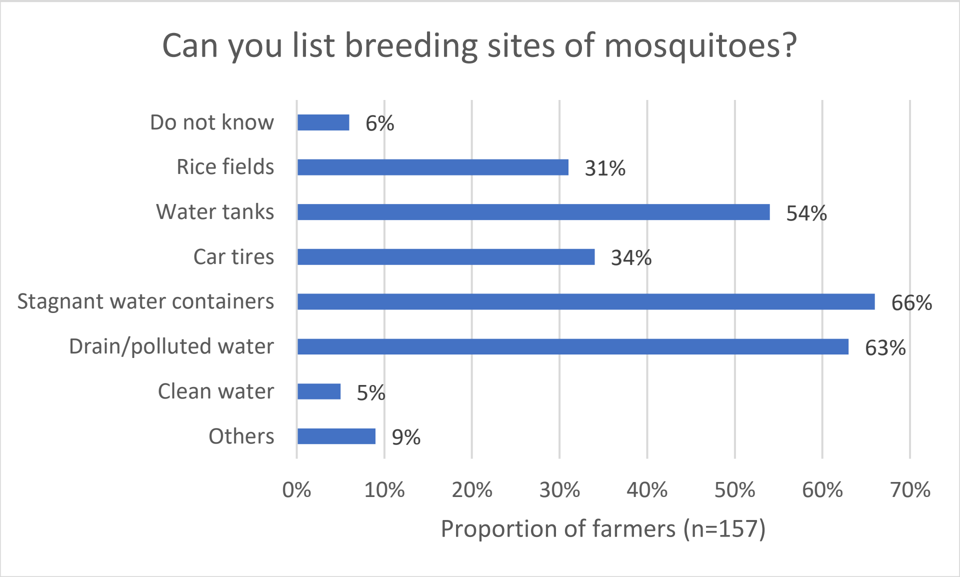

Figure 6. *Proportion of farmers that could list different breeding sites of mosquitoes. Multiple answers were possible.*

Of risk factors for getting mosquito-borne diseases, warm and humid season and stagnant water were the factors most known. Result presented in figure 7 (n=157). More than one risk factor could be chosen. Interviewed with secondary school as the highest level of education could list

1.1 risk factor more than interviewed with primary school or no education together ($p<0.001$). Interviewed with high school/college/university as highest level of education could list 0.7 risk factors more than people with no education/primary school ($p=0.03$).

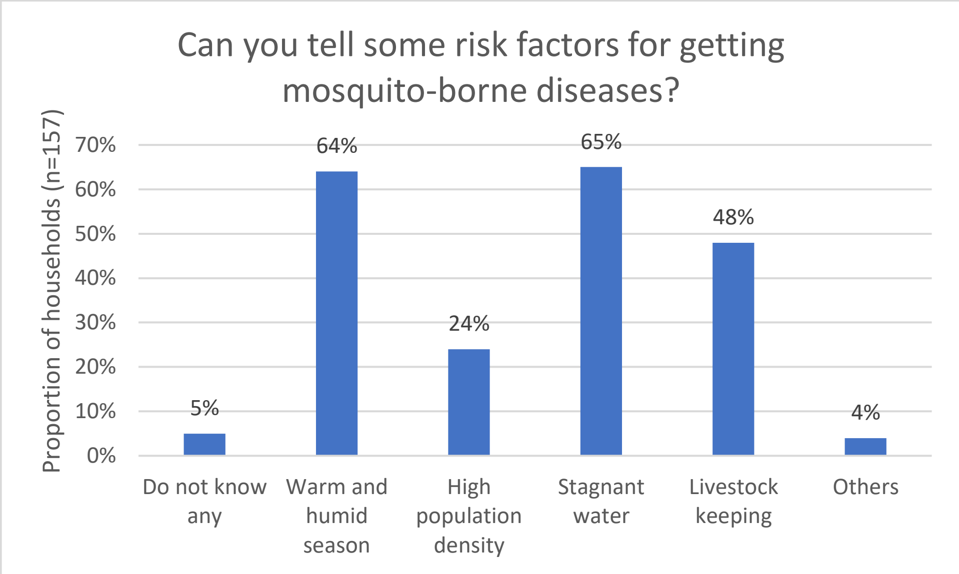

Figure 7. *Proportion of interviewed farmers that could list different risk factors for getting mosquito-borne diseases. Multiple answers were possible.*

Regarding symptoms you can get from mosquito-borne diseases, high fever as well as rashes were the most known symptoms. However, 41% (65 interviewed) could not list any symptoms. Result presented in figure 8. More than one symptom could be listed.

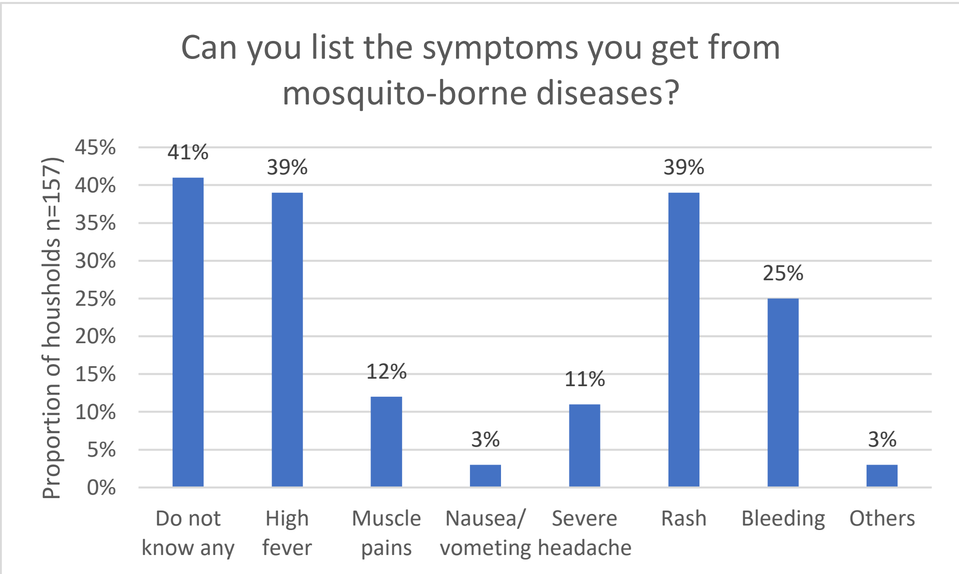

Figure 8. *Proportion of interviewed farmers that could list different symptoms you get from mosquito-borne diseases. Multiple answers were possible.*

All but one of the interviewed were agreed on that the rainy season is the season when mosquito-borne diseases are highest. The last one answered dry season.

There was a difference in the interviewed farmers' awareness about ways to prevent themselves from getting mosquito-borne diseases and which of these preventions they actually used. Mosquito nets were the prevention most known as well as used followed by electric rackets and insecticides. Results are presented in figure 9. There was a significant positive correlation between how many ways to prevent themselves from getting mosquito-borne diseases the interviewed could list and how many they used in practice. For each number of prevention methods they knew, they practiced 0.34 prevention measures more ($p<0.001$). Women practised on average 0.4 more prevention methods than men ($p=0.04$). Regarding frequency of the use of personal protections, 67% (104 interviewed) answered that they use it only during the night. One household use it throughout 24 hours and ten households (6%) only during the day (n=155).

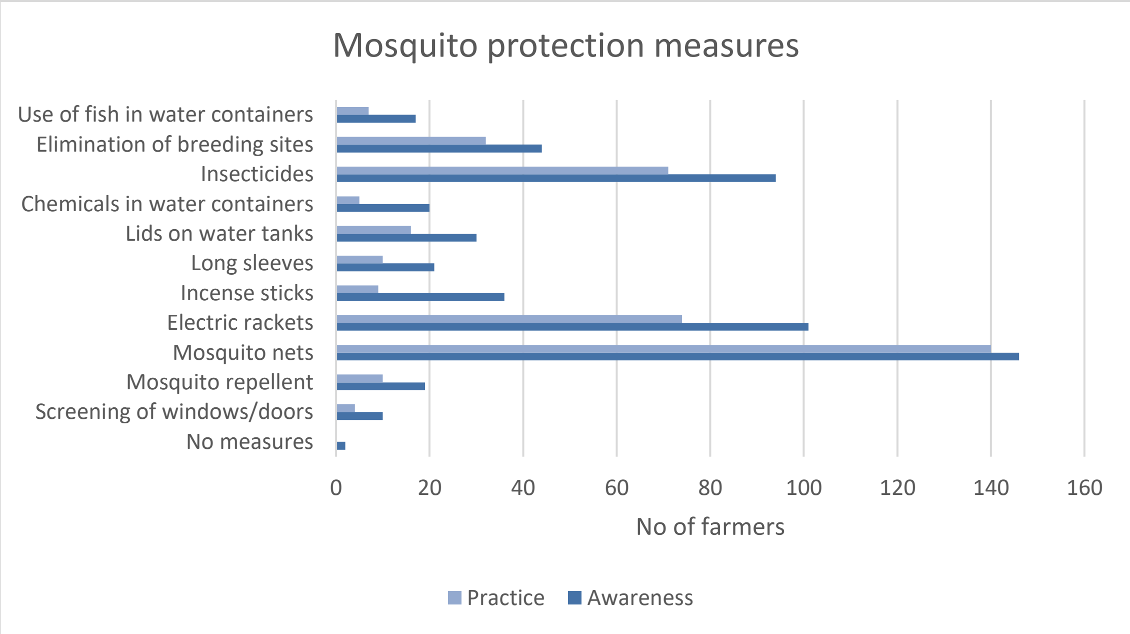

Figure 9. *Farmers practice and awareness of different mosquito protection measures. Multiple answers were possible.*

When combining interviewed farmers with no education and primary school education together in one group, interviewed with secondary school education had 4.5 more knowledge scores than this group. In the group with people with high school and college/university education together, every person had 3.3 more knowledge score than the lowest education group.

Fifty six percent had heard about the term Japanese encephalitis but did not know what it is. Thirty percent knew what Japanese encephalitis is and the rest, 14%, had not heard about the term. Totally 86% either knows about JE or have heard the term. 100% of the interviewed with college/university education either knows about JE or have heard the term. Of the interviewed with no education, 44% did not know what JE is. Ninety six percent of the interviewed who knows what JE is also knows that humans can be affected. Seventy nine percent of the interviewed who have heard the term JE also knows that humans can be affected. Twenty one percent of those who have heard about the term JE did not know that it can affect humans (n=156). No significant difference between level of education were seen. Ten percent of the interviewed knew that pigs can be affected by JE.

The source of information where the most interviewed had heard about mosquito-borne diseases was television (91%), followed by loudspeaker (27%), broadcast (18%) and health staff (13%). Only one interviewed had heard about it from school (n=158).

Four of the interviewed had gotten mosquito-borne diseases in their family. Three of these had gotten malaria and one said to have gotten another mosquito-borne disease than dengue fever, JE or malaria. This person got treated in the public medical system. None of these persons had gotten their disease during the last two years.

DISCUSSION

Farmers using AWD turned out to live in close relation, in the same village, as farmers using conventional rice-growing methods. Additionally, they all lived a few kilometres away from their rice fields. This makes it hard to evaluate the impact of AWD on the number of mosquitoes at the very household. For future projects in the research question it would be of value if whole villages were using AWD to be able to compare it with villages using conventional methods. Another option could be to compare a village with mixed AWD/conventional methods with a village using only conventional. The whole village should hypothetically make a benefit from a significant part of it using AWD. Even though rice fields are a known risk factor for JEV, the fact that the farmers are living far away from their rice fields brings up the question of to what extent the rice fields are a risk compared to breeding sites nearby or at the households or cows, pigs etc. at the households. The one household in this study with cows/buffalos was located in Hai Duong and were using conventional rice-growing methods. This household yielded 200 of the total 732 *Cx. tritaeniorhynchus* mosquitoes, thus having a major impact in the result of the total number of mosquitos and total number of *Cx. tritaeniorhynchus* mosquitoes in Hai Duong as well as for the use of conventional rice-growing methods. Future studies should include better data regarding cow as well as pig density and their location in the village, to be able to do better calculations with these highly relevant factors.

Mosquitoes and larvae from the first field trip in each province were excluded from the calculations in this thesis because of some of these households being visited again during the second field trip and it was at this second trip they were interviewed. Infection rates of JEV in mosquitoes are usually low with less than two per 1000 mosquitoes positive (Lindahl *et al*., 2013). The fact that all mosquito pools turned out negative in this study correlates with these previous known infection rates. Collection of a greater number of mosquitoes would have increased the chance of getting positive pools. Also, there is always a possibility of false negative pools that could have been detected with a more sensitive PCR method.

There are several factors that may have a substantial influence over collected data since the data collection took place over a short period of time and due to most sites only being visited once. These factors include weather variables such as recent or heavy rainfall, time of the year, stage of the rice-growing (e.g. harvest or height of the rice plants affecting the sunlight's ability to reach the water surface below), etc. It would have been of value to collect material several times and during a longer period of time from the same sites, even though this has bias as well (e.g. people in the household learning about breeding sites of the larvae resulting in an elimination of these). The affect of many temporary variables would then be minimized and the sample size would be greater. As an example, recent heavy rainfall prior to our second field trip had made fishes enter the rice field plots from overflooded waters nearby, affecting the possibility to find larvae in the rice field (since fishes eat the larvae). The rainfall during the recent time also affects the amount of stagnant waters in the households. Since the material collection took place in collaboration with another study, some of the households were not randomly selected. Instead they were selected with the criteria of having a dog or not.

In the beginning of the year, African swine fever (ASF) came to Vietnam (Le *et al.*, 2019). Since then it has had a huge impact on the pig population in the country with a large proportion being slaughtered. This were taken in account before the start of this study, but the number of pigs slaughtered turned out to be even higher than predicted, resulting in only seven of the households in the study having pigs. Before ASF, many of the households claimed to have had their own pigs. The impact ASF may have on this study is hard to say, but it could have impact on e.g. number and species of mosquitoes present as well as the number of JEV infected mosquitoes. The affect ASF may have on the presence of JEV from a broader perspective in Vietnam remains to be investigated as well.

Questionnaire

The knowledge about mosquito-borne diseases in general needs to be improved to increase the preventions. Only 31% knew that rice fields are a breeding site of mosquitoes. If this means that they also do not know that it is a risk for getting mosquito-borne diseases, knowledge regarding mosquito-borne diseases would have to increase. If people do not know about the risk from before and do not know about the disease, it will hypothetically work less good as an incentive to start using AWD.

Some of the questions could have been more clearly defined. The questions regarding if there was any stagnant water, pond, water tank etc. near the household gave room for individual differences in interpretation regarding meaning of near. For future surveys this should be defined in e.g. meters prior to the interviews. Some data from the questionnaire were excluded from this thesis because of these divergencies.

The average age of the interviewed can be seen as high. This could be affected by the fact that many families have another job besides farming, resulting in that they are away from home during the day, which was the time of visit. It is possible that households were selected by the criteria of that someone had to be home at the time, resulting in a higher number of households with elderly, retired, people. This could also explain the average low number of children in the households.

CONCLUSION

Even though this study could not prove the hypotheses of a reduction in the number of mosquitoes and JEV carrying mosquitos with the use of AWD, it still does not prove that there is no reduction. There is still a high possibility that AWD have benefits when it comes to reducing the spread of JE but would need trials conducted to allow these comparisons. It is still a highly relevant research subject, not least in step with climate changes and the expanse of the disease to new areas, putting an even higher number of people at risk.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Japansk hjärninflammation (JE) är en sjukdom som drabbar ca 70,000 människor varje år, varav majoriteten är barn. Sjukdomen, som finns i södra och sydöstra Asien samt i norra Australien, är kopplad till hög dödlighet. Nästan 2 miljarder människor bor i områden där sjukdomen finns. I Asien är JE den största orsaken till hjärninflammation. Sjukdomen orsakas av ett virus och sprids med myggor. Risodlingar är det viktigaste stället för dessa myggor att föröka sig på. Grisar är också viktiga i spridningen av viruset eftersom viruset förökar sig i grisarna. En mygga blir smittbärare när den suger blod från en infekterad gris. Myggan kan sedan bita en ny gris eller ibland en människa som då blir infekterad.

Vietnam är ett land i sydöstra Asien med strax över 95 miljoner invånare. Sextiofyra procent av befolkningen bor på landsbygden och nästan hälften av arbetskraften arbetar med jordbruk. Ris är den dominerande grödan och Vietnam är en av de största risexportörerna i världen. Många Vietnameser föder upp grisar antingen för att sälja eller för eget behov. Även i Vietnam är JE den största orsaken till hjärninflammation orsakad av virus.

Alternate wetting and drying (AWD) är en nyutvecklad metod för att odla ris. Metoden sparar vatten och minskar utsläppen av växthusgas från risodlingarna. Risodlingar står för 1,2 % av alla utsläpp av växthusgas i världen. Syftet med den här studien var att undersöka om AWD även har fördelen av att det minskar antalet myggor och antalet myggor som bär på JE-virus. Detta eftersom att risfälten växelvis är torra och myggorna därmed inte kan föröka sig där. AWD skulle alltså kunna ha fördelar både för klimatet och hälsan vilket skulle främja spridning av metoden till större områden.

Även vissa typer av fåglar kan sprida viruset. Fåglar och vanligtvis grisar, blir inte sjuka av viruset. Grisar kan bl.a. abortera men får inte hjärninflammation. Andra djur än grisar och fåglar, inklusive människor, kan inte föröka viruset så mycket att det kan spridas vidare. Människor och dessa andra djur blir smittade av misstag när de är för nära den naturliga viruscykeln mellan grisar, myggor och fåglar. Människor och hästar är de djur som kan få en dödlig hjärninflammation av att bli smittade av viruset. Fåglar är en förklaring till hur viruset kan spridas till nya områden. Grisar är viktiga i smittspridningen till människor eftersom de hålls i nära relation.

Olika arter av myggor är olika bra på att sprida viruset. Den mygga som är viktigast för spridningen av smittan kallas *Culex tritaeniorhynchus*. Det finns dock över 30 olika typer av myggor som har visat sig kunna bära på viruset. Olika typer av myggor har olika smak gällande vilka djurarter de gillar att suga blod ifrån. *Culex tritaeniorhynchus* suger främst blod från kor och i andra hand grisar. Tillgången på de olika djurarterna är avgörande för varifrån de väljer att suga sitt blod. Att hålla grisar och att bo nära risfält är som nämnt riskfaktorer för att drabbas av JE och desto större ytor med risodlingar desto fler *Culex tritaeniorhynchus* myggor. Den totala ytan av risodling har ökat till en mycket stor grad de senaste decennierna i länder där JE finns.

Alla människor utvecklar inte symtom av att bli smittade av JE. Allt från 1 av 25 till 1 av 1000 får symtom. Symtom kan vara allt ifrån att likna influensa till onormalt beteende och minskad medvetenhet. Dödligheten är 30 % hos de drabbade som blir inlagda på sjukhus. Ungefär hälften av de som överlever får neurologiska följdsjukdomar. Säker diagnos ställs vanligtvis med blodprov om det finns möjlighet till detta. Det finns inget botemedel för sjukdomen, men det finns vaccin. Världshälsoorganisationen (WHO) rekommenderar att det ska finnas vaccinationsprogram i alla områden där JE är ett problem för folkhälsan.

I den här studien fångades myggor och mygglarver in i två olika provinser (Hai Duong och Thai Binh) utanför Hanoi i norra Vietnam. De fångades i risodlande bönders hushåll, där hälften av bönderna använde AWD-teknik och hälften använde en konventionell risodlingsmetod. Förekomsten av larver i risfälten undersöktes också. Myggorna och larverna räknades och sorterades efter art, varpå de analyserades för förekomst av JE-virus. Utöver detta genomfördes en enkätstudie gällande dessa bönders kunskap om myggburna sjukdomar, JE och vad de gör för att undvika att bli smittade. Totalt intervjuades 160 hushåll. Tre resor gjordes till varje provins med en månads mellanrum.

Totalt fångades 2,072 myggor och 1,725 larver. Ingen statistisk signifikant skillnad kunde ses i totalantalet myggor mellan de olika provinserna. Inte heller mellan AWD och konventionell risodling. Gällande art så hade provinsen Hai Duong en större andel av myggan *Culex tritaeniorhynchus* än Thai Binh. Myggorna analyserades för att hitta JE-virus, men alla testade negativt.

För varje antal personer som bodde i hushållen ökade antalet myggor med 1,3. För varje gris i hushållet ökade antalet med 1,6 och för varje ko med 631 av myggan *Culex tritaeniorhynchus*. Totalt var det sju hushåll som hade grisar och ett hushåll som hade kor. Åttiotvå procent av alla intervjuade hade hört talas om sjukdomar som överförs från myggor till människor men endast 25 % hade hört talas om sjukdomar som överförs från myggor till grisar. Desto högre utbildningsnivå den intervjuade hade, desto fler hade hört om sjukdomar som överförs från myggor till människor. Endast 31 % visste att risodlingar är en plats där myggor förökar sig. Alla hushåll utom ett visste att myggburna sjukdomar är som högst under regnsäsongen. Trettio procent visste vad JE är, 14 % hade aldrig hört talas om det och 86 % hade hört termen men visste inte vad det var. Av de personer som inte hade någon utbildningen var det 44 % som aldrig hade hört talas om sjukdomen. Endast 10 % visste att grisar kan bli smittade av JE. I fyra av hushållen hade någon person i familjen någon gång blivit drabbad av en myggburen sjukdom, tre av dessa hade fått malaria.

Bönder som utövade AWD och bönder som utövade konventionella risodlingsmetoder visade sig bo i nära relation till varandra i byar några kilometer från deras risodlingar. Detta är en av förklaringarna till varför det inte gick att visa någon skillnad i antal myggor mellan de två olika metoderna. Framtida forskning i frågan bör fokusera på risodlingar/byar där metoderna inte är blandade. Alternativt jämföra områden med blandad utövning med områden med endast konventionell, då hela områden hypotetiskt borde gynnas om en större andel utgörs av AWD. Även fast risodlingar är en känd riskfaktor för att drabbas av JE är frågan om till vilken del det är en risk i relation till att t.ex. hålla kor och/eller grisar och att ha andra ställen i nära relation där myggorna kan föröka sig (t.ex. i stillastående regnvatten på hushållstomten). Framtida forskning i frågan bör även samla in större material under en längre tid för att minska påverkan av bl.a. väder och vilket stadium risodlingen är i. Vietnam hade under tiden för studien drabbats hårt av sjukdomen afrikansk svinpest, vilken gjort att många grisar slaktats ut, vilket möjligtvis haft inverkan på resultatet av studien.

Även då den här studien inte kunnat bekräfta hypotesen, att AWD minskar antalet myggor och antalet myggor som bär på JE-virus, utesluter det inte att det finns ett samband. Det är ett aktuellt forskningsämne med många potentiella fördelar som behöver undersökas vidare, inte minst i takt med att klimatet förändras, sjukdomen sprids till nya områden och fler utsätts för risken att drabbas.

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