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An epidemiological and virological study on mosquito-borne flavivirus transmission

The role of urban livestock keeping and the risk for mosquito-borne zoonotic diseases in humans in Hanoi city, Vietnam

THANG NGUYEN-TIEN

Abstract

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Mosquito-borne diseases (MBDs), which are transmitted by the bite of mosquitoes to humans, are causing high morbidity and mortality worldwide and are emerging in many tropical cities. Urban livestock keeping is still growing in cities around the world, which can itself contribute to increasing the mosquito populations. In addition, the animals may also serve as reservoirs for zoonotic MBDs, which increase the risks for humans. In this thesis, knowledge and practices related to MBDs were assessed in 513 households, with and without livestock. The perceptions of the community and health care sector were explored, with a focus on the association of MBDs and livestock keeping in Hanoi city of Vietnam. Generally, the participants had relatively low awareness on MBDs prevention and control. Those who had better knowledge did also use more adequate preventive practices against MBDs ($p<0.001$). An entomological survey was performed to explore how livestock keeping in and around a large city was associated with the presence of mosquitoes and their risk for spreading flaviviruses. The findings indicated that the distribution of vector populations in Hanoi city was heterogeneous, and that livestock played a role in influencing this. Households with better preventive practices reduced the presence of mosquitoes. Pig keeping particularly lead to more mosquitoes – especially of the *Culex* genus. 12,861 adult mosquitoes were collected and pooled into 385 pools, whereof one pool of *Culex tritaeniorhynchus* was found positive for Japanese encephalitis virus (JEV) by a pan-flavivirus qPCR. Phylogenetic analysis indicated that the detected virus belonged to genotype I. A hospital-based study with 98 dengue patients and 99 non-dengue patients was conducted to investigate risk factors of dengue infection and dengue virus (DENV) variants circulating in Hanoi. Age group and living district were the identified risk factors that correlated with dengue status. Serum and blood samples were analysed by a panflavivirus qPCR. The results showed that DENV serotypes 1, 2, and 4 were co-circulating during this outbreak, of which DENV serotype 1 was the predominant genotype.

This thesis provides insight into the risk of flavivirus-related diseases such as dengue fever and Japanese encephalitis for those living in Hanoi metropolitan city, Vietnam, and the links between mosquitoes, animals and humans. Expanded and effective vector control measures and vaccination programs need to be taken to prevent the transmission of mosquito-borne flavivirus due to the significant dispersion of *Culex* and *Aedes* mosquitoes.

Keywords: urban flavivirus, mosquito-borne diseases, livestock keeping, urban Vietnam, Southeast Asia.

Thang Nguyen-Tien, Department of Medical Biochemistry and Microbiology, Box 582, Uppsala University, SE-75123 Uppsala, Sweden.

"It is during our darkest moments that we must focus to see the light." - *Aristotle*

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Nguyen-Tien T**, Pham-Thanh L, Vu TD, Tran HS, Vu TL, Bui NV, Bui NA, Hoang DT, Vu TT, Nguyen-Viet H, Magnusson U, Lundkvist Å, Lindahl FJ. Knowledge and practice on prevention of mosquito-borne diseases in livestock keeping and non-livestock keeping communities in Hanoi city, Vietnam: A mixed-method study. *PLoS One*. 2021;16(2 February):1–21. (This paper will also be part of the thesis of Pham-Thanh L)

II. **Nguyen-Tien T**, Bui NA, Ling J, Tran-Hai S, Pham-Thanh L, Bui NV, Dao DT, Hoang TT, Vu TL, Tran VP, Vu TD, Lundkvist Å, Nguyen-Viet H, Magnusson U, Lindahl FJ. The Distribution and Composition of Vector Abundance in Hanoi City, Vietnam: Association with Livestock Keeping and Flavivirus Detection. *Viruses* 2021, *13*, 2291. (This paper will also be part of the thesis of Pham-Thanh L)

III. **Nguyen-Tien T**, Do DC, Le XL, Dinh TH, Lindeborg M, Nguyen-Viet H, Lundkvist Å, Grace D, Lindahl FJ. Risk factors of dengue fever in an urban area in Vietnam: a case-control study. *BMC public health*. 2021 Dec;21(1):1-3.

IV. **Nguyen-Tien T**, Ling J, Lindeborg M, Dao DT, Pham-Thanh L, Bui NA, Bui NV, Do DC, Le XL, Dinh TH, Nguyen-Viet H, Lundkvist Å, Grace D, Lindahl FJ. Clinical symptoms and dengue virus detection in adult patients in Hanoi city, Vietnam. *Manuscript.*

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Additional publications

Paper not included in thesis:

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Jakobsen F, **Nguyen-Tien T**, Pham-Thanh L, Bui NV, Nguyen-Viet H, Tran HS, Bui NA, Lundkvist Å, Lindahl FJ. Urban livestock keeping and dengue in urban and peri-urban Hanoi, Vietnam. *PLoS Negl Trop Dis*. 2019.

Chapot L, **Nguyen-Tien T**, Pham-Thanh L, Nguyen-Viet H, Craven L, Lindahl FJ. A Mixed-Methods Approach to Understanding Knowledge of Mosquito-Borne Infections and Barriers for Protection in Hanoi, Vietnam. *Trop Med Infect Dis*. 2020 May;5(2):66.

Ha TV, Kim W, **Nguyen-Tien T**, Lindahl FJ, Nguyen-Viet H, Thi NQ, Nguyen HV, Unger F, Lee HS. Spatial distribution of Culex mosquito abundance and associated risk factors in Hanoi, Vietnam. *PLoS Negl Trop Dis*. 2021 Jun 21;15(6):e0009497.

Pham-Thanh L, **Nguyen-Tien T**, Magnusson U, Bui-Nghia V, Bui-Ngoc A, Le-Thanh D, Lundkvist Å, Can-Xuan M, Nguyen-Thi Thu T, Vu-Thi Bich H, Lee HS, Nguyen-Viet H, Lindahl FJ. Dogs as Sentinels for Flavivirus Exposure in Urban, Peri-Urban and Rural Hanoi, Vietnam. *Viruses*. 2021 Mar;13(3):507.

Contents

Abbreviations

Bp	Base pairs
CI	Confidence interval
Ct	Cycle threshold
DENV	Dengue virus
DF	Dengue fever
IDI	In-dept interview
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
KAP	Knowledge, attitude, practice
K&P	Knowledge and practice
MAFFT	Multiple Alignment using Fast Fourier Transform
MBDs	Mosquito-borne diseases
OR	Odds ratios
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
ZIKV	Zika virus

Introduction

Emerging vector-borne diseases, especially mosquito-borne diseases, are constituting an increasing threat to human health globally [1]. The majority of MBDs are zoonoses for which humans are often incidental or dead-end hosts [2]. In the last decades, the emergence of MBDs has risen dramatically and their geographical distribution has expanded, leading to an increasing number of epidemics worldwide [3]. MBDs previously thought to be under control, such as dengue fever (DF) and Japanese encephalitis (JE) are now re-emerging and the introduction of viruses such as Zika virus (ZIKV) in new areas poses a global public health threat [3,4]. Some MBDs are widely spread across the world and are responsible for an important social and economic burden. In Southeast Asia, changes in climate and human activities are providing favourable conditions for the dispersal and establishment of arboviral diseases: rapid population growth and urbanization, lacking sanitation and drainage, change in land use, increased movement of people and international air travel have led to closer contact between humans and vectors and facilitated their spread [5]. No vaccines are yet available for the majority of MBDs. Therefore, prevention relies mainly on vector control through environmental management and change of behaviour [6]. However, the effectiveness of such methods may be limited due to lack of understanding, funding, and/or engagement from the authorities and communities [7].

Keeping livestock in urban areas is a common way to provide urban inhabitants with perishable high value food products and to create opportunities for livelihoods [8]. Increasing the number of livestock in the growing cities means that mosquitoes have more feeding sources. They are adapting and finding more urban breeding habitats. The livestock can become potential blood sources for some mosquitoes. Moreover, livestock keeping may also cause more water sources to be around when feeding water for the animal or storing water to clean the animal shelter that can create more mosquito breeding sites. It is, therefore, hypothesized that livestock keeping may increase vector populations since the animals may serve as blood sources for mosquitoes and reservoirs for pathogens, leading to increasing risk of mosquito-borne zoonotic infections of humans [9,10].

However, there is only limited research on the association between livestock keeping and MBDs globally, as well as in Vietnam. In order to address

this gap, this thesis work was conducted in Hanoi city, Vietnam, to unravel the hypothesis in multiple approaches.

Mosquito-borne flaviviruses

Flavivirus is a genus within the Flaviviridae family, which members cause disease in animals and humans. There are over 70 different flaviviruses described, with the mosquito-borne flaviviruses accounting for more than half. Flaviviruses are responsible for a variety of common well-known diseases such as JE, West Nile fever, Yellow fever, DF, Zika etc [2,11,12]. In Southeast Asia and Vietnam, JEV, dengue virus (DENV) and ZIKV have been the most prevalent flaviviruses in recent decades.

JEV is transmitted by several *Culex* mosquito species with the primary vector being *Culex tritaeniorhynchus*. In the transmission cycle, water-birds and pigs act as virus maintenance and amplification hosts, respectively, while humans only serve as dead-end hosts [13]. JEV is the leading cause of viral encephalitis in Asia, causing over 68,000 cases annually [14]. The disease affects mostly children in rural rice-growing areas, which provide ideal mosquito breeding sites [2]. Infections are usually asymptomatic. Less than 1% of the cases develop clinical encephalitis, which is characterized by a Parkinsonian syndrome including mask-like faces, tremor, hypertonia and abnormalities of movement. Among the clinical JE cases, 30% are fatal and 50% lead to severe neurological sequelae responsible for sometimes lifelong disabilities. There is no antiviral treatment available [2,15]. Immunization through vaccination is the only sustainable method for long-term prevention. In complement to vector control, vaccination has contributed to decrease the risk of infection in endemic areas and is now part of the routine immunization programs in several Southeast Asian countries, including Vietnam [2].

DENV is transmitted primarily by *Aedes aegypti* and secondarily by *Aedes albopictus* [16]. It is one of the rare arboviruses that have fully adapted to humans and no longer requires an animal reservoir for transmission [3]. DENV mosquito vectors are adapted to peri-domestic urban habitats where they breed in water storage containers. They bite during daytime, therefore traditional vector control methods such as nets have limited effectiveness. Weather components such as rainfall and temperature have been shown to have a strong impact on the distribution, development and survival of Aedes [17]. As a result of climatic, demographic and societal changes, their abundance and geographical range are expected to increase in the near future, leading to more frequent and severe epidemics [18,19]. There are four different DENV serotypes (DENV1-4). Recently, another type called DENV-5, related to a sylvatic transmission cycle has been described [20], leading to the concern about the ineffectiveness of dengue control. Similar to JE, most dengue infections are asymptomatic. Classical DF usually causes febrile mild illness but,

in some cases leads to severe forms, called dengue shock syndrome and dengue haemorrhagic fever. Treatment is only symptomatic, and severe cases require hospitalization [2]. Since the symptoms are non-specific, a definitive diagnosis requires laboratory testing and is rarely obtained in practice.

ZIKV is also transmitted by *Aedes* species. Similar to DENV, it can sustain transmission through a human-mosquito-human cycle without depending on an animal reservoir. It can also spread vertically in humans from mother to foetus and through sexual intercourse. Transmission through blood transfusion has been documented [21]. The first major outbreak was described in Micronesia in 2007. Prior to that, it had only been detected in Africa and Southeast Asia. It was declared a Public Health Emergency of International Concern by WHO following the large outbreak in Brazil in 2015. Subsequent studies showed that the virus had been circulating in Southeast Asian countries for many years, including Vietnam [5]. ZIKV infections are asymptomatic or cause mild disease in most cases, but has been associated to neurodevelopmental birth defects, including microcephaly. There is no treatment and vaccines are still under development. Control methods focus on reducing exposure to vectors, with particular attention to pregnant women [21].

Urban epidemiology of flaviviruses in Vietnam

Vietnam is a tropical country and a hot spot for MBDs. DF, Zika and JE are among the most common MBDs in humans and the viruses can co-exist in urban settings. The urban epidemiology of JEV has been studied in different parts of Vietnam. Several potential vectors for JEV have been identified in entomological surveys, and the presence of virus detected by reverse transcription - polymerase chain reaction (RT-PCR). JEV has the potential to rapidly emerge in urban communities, particularly when pig keeping is practiced in peri-urban areas of cities, as frequently occurs in Vietnam. However, human cases of JE have been increasingly controlled by an expanded immunization program [22].

Southeast Asia accounts for more than 70% of global dengue morbidity and mortality, and Vietnam is one of the countries with the highest burden [23]. DENV is also the most widespread flavivirus in Vietnam. All DENV serotypes are circulating and are regularly transmitted in urban and semi-urban areas throughout the country with highest burden in larger cities of the Northern, Central and Southern regions [22].

ZIKV was detected in the Northern region of Vietnam already 1954 [24], but it seems to have been re-emerging only in urban areas of the Central, Central Highlands and Southern regions during recent years, especially in the Ho Chi Minh city, where migration and huge international travel are presented [22]. Other flaviviruses, such as Quang Binh virus, circulate in Vietnam but have so far not been investigated in terms of urban transmission [22].
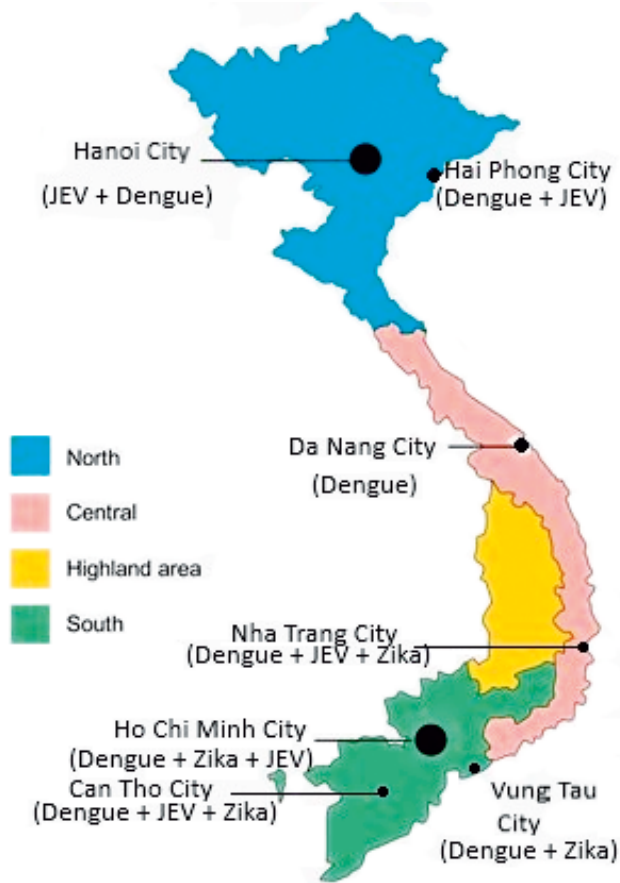
Figure 1 - Distribution of three prevalent flaviviruses in large cities in Vietnam (retrieved from [22])

Community awareness on MBDs

Many studies have assessed the knowledge, attitude and practices (KAP) of communities about MBDs. One earlier study carried out in India by Ravi Kumar and co-workers [25] explored the community perception on MBDs in both urban and rural areas. The findings revealed relatively low level of knowledge on MBDs in the community and indicated a need of interventions related to social mobilization and communication for changes of behaviour. Studies by Anand and co-workers [26] and Boratne and co-workers [27] focused on assessing the knowledge, attitude, and use of personal protective measures against MBDs in an urban capital and a coastal area of India. Anand and co-workers' study also revealed gaps in knowledge and use of personal protective measures to prevent MBDs [26], but the study by Boratne and co-

workers found that the use of personal protection methods was low only in some specific groups of the population [26]. However, both studies recommended an improvement of information/education/communication activities to raise the public knowledge and enhance community participation in prevention and control of MBDs [26,27].

In Malaysia, a tropical country in Southeast Asia where mosquito-borne diseases are endemic, Aziz Shafie and co-workers [28] conducted a community-based survey to assess the level of knowledge, awareness, and practices toward MBDs among various ethnic groups. The results revealed that the respondents generally had a low level of knowledge in relation to MBDs - including transmission, signs and symptoms, prevention, and treatment of the disease. A recent study performed by Imelda and co-workers [28] in Brazil tested the level of knowledge and risk perception for dengue, Chikungunya and Zika infections in two groups of healthcare personnel and the general population. There was a gap in KAP, and risk perception of diseases transmitted by *Aedes aegypti* in all groups. Also, the results depicted the significant differences between these two groups in KAP, and risk perception of those diseases. Chao Wu and co-workers [29] implemented a cross-sectional study to examine behaviours related to MBDs in China-Laos border areas. This study revealed different practices between different ethnic minority groups and different socioeconomic groups, in relation to MBDs including rearing pigs, bed net use and repellent use. Based on these results, they recommended different solutions for the prevention of MBDs for each group. Hence, much evidence has been provided to indicate that low level of knowledge and poor practice of preventive measures against MBDs in communities. And low awareness on MBDs may increase its morbidity [30]. Improvement of knowledge and practices (K&P) can be one of the key measures to prevent the wide transmission.

Urban livestock keeping

Urban agriculture is growing globally for different reasons. People are continuously moving from rural to urban areas in the context of the rapid urbanization process, contributing to the dense human population in these cities. Their basic demands include fresh food products originating from plants, seafood and animals. Therefore, urban agriculture is a solution for food supplying in the urban cities [31]. In addition, it is a way to adapt to climate change since it can play a key role in greening the city through reusing the organic waste [31,32]. Open spaces in the urban areas are also used productively with agricultural activities while people can generate income from these job opportunities [33].

Livestock keeping is a part of urban agriculture that provides vital sources of nutrition such as meat and dairy for the dweller of the cities [8,34]. In tropical countries with lacking infrastructures, it can be impossible to transport

animal-source foods over long distances. Also, rearing the livestock will provide important jobs for urban inhabitants of whom many are originally farmers and poor people. Hence, it is obvious that livestock keeping creates very important profits and incomes [8,35,36].

However, there may be negative effects in addition to the benefits of rearing animal in the urban areas, and city planners as well as keepers should not ignore the risks and problems. For example, livestock waste/manure management is a big concern, not only for the keepers, but also for their neighbours and the surrounding community. The solid and liquid waste generated by the livestock keeping systems can also affect the surrounding environment, water and land resources [8,35]. Another constraint of livestock keeping is how to manage and treat the diseases that livestock may have [37,38]. Since animals may share the living environment with people to save space in densely populated cities, they may contribute to health risks for the livestock keepers and the community through zoonotic diseases, including vector-borne diseases, water-borne diseases, urine-borne diseases or food-borne diseases [8,35,38].

The connection between livestock – mosquitoes – human

Besides the advantages for high value animal-source food products; livestock keeping is potentially increasing the risk for human infections with mosquito-borne pathogens. In Ethiopia and Pakistan, study findings indicate that the presence of cattle or goats near to homes tends to increase the human-biting rate of *Anopheles* mosquitoes [39,40]. Other studies also highlight that keeping goats and/or other medium-sized livestock - such as sheep and pigs - may contribute to an increased risk of non-zoonotic MBDs, such as malaria, by increasing the number of vectors [41,42]. One study in Vietnam found that urban pig keeping was associated with an increase in the number of JEV vectors; in this study, Lindahl and co-workers [43] showed that the number of pigs in households was correlated with the increase in the numbers of *Cx. tritaeniorhynchus* and all of the mosquitoes in the *Cx. vishnui* subgroup, while the number of people in the households increased the number of *Cx. quinquefasciatus*. Collected *Culex* mosquitoes were found to be positive for JEV, belonging to both genotypes I and III, while 100% of the pigs in the study were found to have JEV-reactive antibodies [9]. Another study in Vietnam indicated that having an animal shelter correlated to a higher incidence of DF [30]. However, a recent small study of Jakobsen et al. [44] conducted in Hanoi city, Vietnam, could not find evidence that households with livestock were at higher risk of getting DF as compared to non-livestock households. Therefore, the connection between livestock and mosquitoes and human is a debatable issue that need to be unravelled.

Aims of this thesis

This thesis strives to increase the understanding of urban disease epidemiology in Hanoi metropolitan city, Vietnam, where multiple flavivirus MBDs are occurring simultaneously and clarify on how urban livestock is contributing to these zoonotic risks.

Specific aims

- To assess the level of knowledge and practice of urban dwellers in livestock keeping and non-livestock keeping communities and explore the perception from healthcare sector about MBDs and livestock keeping in Hanoi city, Vietnam.

- To evaluate the distribution and composition of mosquitoes in Hanoi city and explore the association between urban mosquito population and livestock keeping.

- To detect flaviviruses present in collected urban mosquitoes.

- To identify the risk factors and describe the molecular epidemiology of dengue virus by investigating febrile patients at a national hospital in Hanoi city, Vietnam.

Methods and materials

Definitions

In this thesis, the living districts of participants were categorized into three different areas: central urban, which comprises the inner districts of old Hanoi where no livestock is kept; peripheral that are expanded districts of Great Hanoi where some livestock are kept, and peri-urban that comprises suburban districts where many livestock are kept.

In study I, a household with livestock was defined as a household having at least one larger livestock species (pig, cattle or larger); or at least 5 smaller food-producing animals (chicken, duck, goose, rabbit), meaning at least 0.05 tropical livestock units [45].

In study II, the criteria for DF confirmation were based on the dengue case definition in Decision 458/QD-BYT and its guideline issued by the Vietnam Ministry of Health in 2011. The content of the guideline includes the clinical and sub-clinical characteristics of DF; diagnosis and treatment of DF applying for all healthcare facilities in Vietnam. Patients, suspected for DF on clinical symptoms, were confirmed by a rapid test for dengue virus NS1 antigen during the first five days, or dengue-specific IgM after the fifth day of the disease [6,46]. The case group (dengue patients) included in-patients who were diagnosed with dengue and confirmed positive by the rapid tests for NS1 antigen or IgM antibodies. The control group (non-dengue patients) included out-patients or in-patients who were not diagnosed with DF and confirmed negative by the rapid test according to the case definition above.

Study sites of Hanoi metropolitan city

Hanoi is the capital city which is located at Red River Delta in the northern region of Vietnam. It is one of the two economic hubs of the country that has a considerable rate of urbanization. Before 2008, old Hanoi city was divided into nine urban and five peri-urban districts. After merging some areas of surrounding provinces, the new Hanoi, now called "Great Hanoi", has increased to 12 urban districts, 17 peri-urban districts and one district-levelled town. The population of Hanoi metropolitan city is now more crowded and reaching around seven million people [47]. Hanoi city has a warm and humid sub-tropical climate with four seasons.
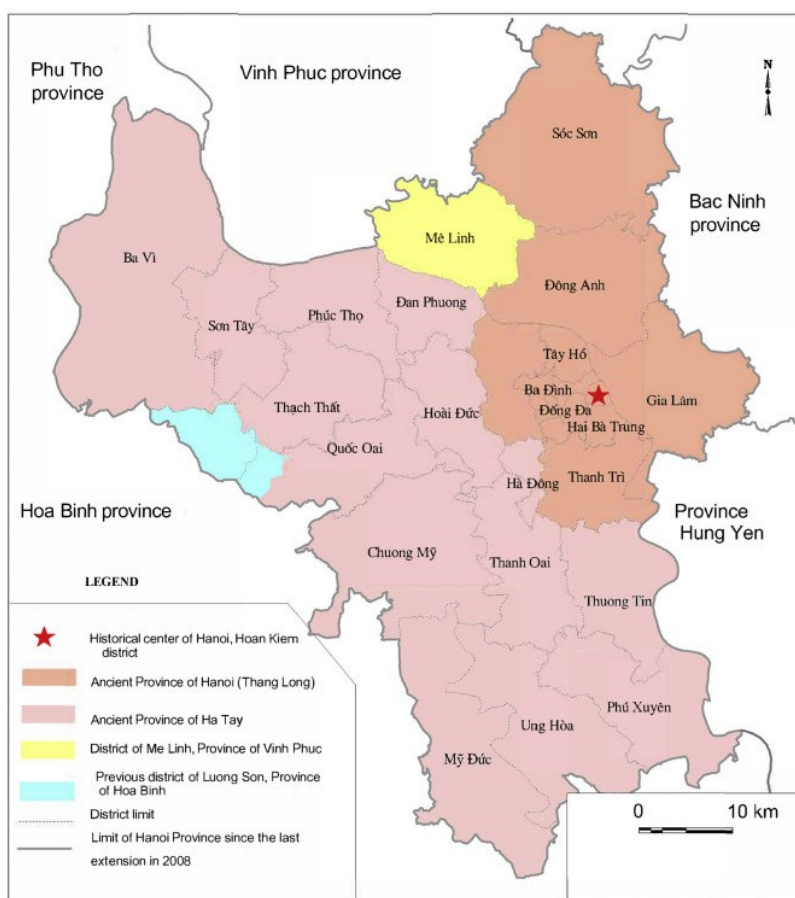
Figure 2 – Map of Great Hanoi (retrieved from [47])

With the favourable characteristics of climate and massive population growth along with unplanned urbanization, Hanoi city is an endemic area for MBDs including DF and JE. Besides, according to the General Statistics Office, the number of livestock in Hanoi is populous, with around 157,500 cattle, 1,635,900 pigs and 25,620,000 poultry reported in 2018 [48]. Livestock rearing can be a risk factor of vector growth in the community.

Previously, Hanoi had a low incidence of dengue infections [49], however, the morbidity of dengue has increased during recent decades with outbreaks occurring in more frequent cycles. In 2009 and 2017, Hanoi city recorded the two largest dengue outbreaks in both urban and suburban districts [50–52]. In addition, JEV is circulating in this city since cases of JE are still reported, especially in children who have not been vaccinated. The statistical data revealed that there has not been any infection with other flavivirus reported so far in Hanoi [53]. For a better understanding of the methods and materials used in two studies, a summary is presented in the table below.

Table 1 Overview of methods used in two studies

	Cross-sectional study I		Study II
	First objective	**Second objective**	
Research objectives	Assess K&P of urban livestock-keeping and non-livestock-keeping communities Explore perceptions of healthcare sector about MBDs and livestock keeping	Evaluate the distribution of mosquitoes in Hanoi city Explore association between urban mosquito populations and livestock-keeping. Detect flaviviruses present in mosquitoes.	Identify the risk factors and describe the molecular epidemiology of DENV by investigating febrile patients in a national hospital in Hanoi city, Vietnam.
Type	Community-based K&P survey	Community-based entomological survey	Hospital-based survey
Design	Cross-sectional	Cross-sectional	Case-control
Settings	6 selected districts	6 selected districts	Department of Infectious Diseases, Bach Mai hospital
Research population	People above 18 years old in households with and without livestock Representative from Hanoi Center for Disease Control and 3 district health centers	Mosquito and larvae collected outdoor and indoor at the households	In-patients and out-patients of Department of Infectious Diseases and their blood samples

	Cross-sectional study I		Study II
	First objective	**Second objective**	
Time	September – December 2018	September – October 2018	September 2018 – January 2019
Sample size	513 households	Mosquito and larvae collected in 513 households	98 dengue patients and 99 non-dengue patients
Data collection methods	Face-to-face interviews In-depth interviews	BG sentinel traps and CDC light traps Larvae collection	Face-to-face interviews Medical records
Lab analyses	None	Pan-flavivirus RT-PCR Long non-coding RNA sequencing	Pan-flavivirus RT-PCR Sequencing
Data analyses	Cronbach Alpha for questionnaire consistency Negative binomial regression model. Content analysis	Zero-inflated negative binomial regression model Phylogenetic analysis	Cronbach Alpha for questionnaire consistency Multivariable logistic regression

Study design

In study I, cross-sectional design was employed in community setting. Mixed methods were applied for the first objective. The second objective was an entomological investigation where mosquitoes and larvae were collected at the households. *Study II* was hospital-based research using case-control design.

Sample size and sampling

In study I, two random transects were created from the central urban to peri-urban districts in Hanoi city map. Six districts from the transects were identified including two central urban districts without livestock (*Ba Dinh* and *Cau Giay)*, two peripheral urban districts with some livestock (*Ha Dong* and *Bac Tu Liem)*, and two peri-urban districts with large livestock populations (*Chuong My* and *Dan Phuong)*. In the selected six districts, the households were selected using randomly generated GPS-points in QGIS software. From each GPS-point, three livestock households were identified and approached in three different directions within a radius of 2 km, and three other non-livestock households nearby. Thus, six households were chosen for each randomly selected point. In total, **513 households** were visited in the quantitative and entomological survey as indicated in Table 2 below:

Table 2 – Sample size of study I

District	Number of households	
	With livestock	**Without livestock**
Peri-urban districts	**129**	**80**
Chuong My	67	35
Dan Phuong	62	45
Peripheral urban districts	**104**	**98**
Ha Dong	55	45
Bac Tu Liem	49	53
Central urban districts	**0**	**102**
Cau Giay	0	51
Ba Dinh	0	51
Total	**233**	**280**

For the qualitative survey, amongst 513 households, five households with livestock and five households without livestock in six districts were conveniently chosen from those included in the quantitative survey, based on their availability for person-to-person in-depth interviews (IDIs). We also had IDIs with one representative from the Department of Vector-borne Disease of Hanoi Centre of Disease Control and three representatives from the health sector

who worked in communicable diseases and/or vector surveillance in three categorized district health centers including *Ba Dinh*, *Ha Dong* and *Chuong My*.

In study II, 98 cases and 99 control patients participated in the study. They were in- and out-patients of the Department of Infectious Diseases at the Bach Mai hospital who were recruited by the doctors depending on their health status and if the patients were volunteering.

Data collection and instruments

Data collection and vector collection of *study I* were performed from September to December 2018. Questionnaire survey and vector collection were conducted in parallel from September to October 2018 while qualitative investigation was implemented later in December 2018.

Questionnaire data was collected by interviewing the household owner or one adult (above 18) living permanently in the household with a structured questionnaire. The questionnaire included four sections with a number of multiple-choice and open-ended questions: (i) Demographic characteristics of the respondent, (ii) livestock information; (iii) awareness about MBDs, (iv) sources of information that participants have heard about the MBDs. The face-to-face interviews took place in the households and lasted around 20 minutes.

Qualitative interviews were conducted using two self-constructed IDI guidelines and tape-recorded with participation's permission. The recorded files were re-listened to check the quality and transcribed after that.

At 513 households, non-baited CDC light traps were used outdoors, while both BG-Sentinel traps with lures and non-baited CDC light traps were used indoors. In households without livestock, the outdoor collection was performed in the garden or yard. Before leaving the households, the traps were set and run overnight. Mosquitoes were collected in the early morning next day. The samples were labelled and put in a cool box and brought to the Vietnam National Institute of Hygiene and Epidemiology for storage at −80°C. Larvae was collected by searching all containers in the households with sweep nets to catch mosquito larvae. The collected mosquitoes were counted and identified by species and gender, while larvae were also identified to genus level according to recommended keys [54].

In study II, the questionnaire was developed in Google form with three main components of demographic, household information and knowledge, attitude and practices (KAP) related to mosquitoes and DF. Two trained data collectors used a tablet to interview the patients during 15 to 25 minutes.

Laboratory analysis

For the second objective of study I, the mosquito samples were grouped into 385 pools of a maximum of 48 individuals by species and collection site. Pools of mosquitoes were homogenized, and then total RNA was extracted using a RNeasy Mini Kit (Qiagen, Hilden, Germany).

A pan-flavivirus RT-qPCR was used following the modified protocol from Patel et al. [55], with two different forward primers and one reverse primer (Table 3). As positive controls, RNAs extracted from a local DENV-1 strain (D7709, Vietnam) and the JEV Nakayama strain (genotype III) in dilutions of $10^{-1} - 10^{-3}$ were used.

The QuantiTect SYBR® Green RT-PCR kit was used according to the manufacturer´s instructions (Qiagen-204245, Germany). In each reaction, 2× QuantiTect master mix, 0.4 µM of each primer, 1× QuantiTect RT mix, RNase-free H2O, and 5 µL of RNA creating a total volume of 25 µL were included. The PCR amplification conditions were as follows: reverse transcription at 50°C for 30 minutes, initial activation at 95°C for 15 minutes, followed by 45 cycles of denaturation at 94°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds.

Table 3. Names and sequences of the primers used in the pan-flavivirus qPCR.

Primer	*Sequence*
Flavi all S	TACAACATgATggggAARAgAgARAA
Flavi all S2	TACAACATgATgggMAAACgYgARAA
Flavi all AS4	gTgTCCCAGCCNgCKgTRTCRTC

A sample was considered positive if both duplicated samples had a cycle threshold (Ct) value below 40 and with correct melting temperatures (Table 4). The cut-off points were validated based on the protocol of Patel et al. [55] at the Zoonosis Science Centre at Uppsala University. Gel electrophoresis was carried out with the positive sample to confirm whether the correct product had been amplified in the pan-flavivirus RT-qPCR. A 2% agarose gel was prepared with 1× TAE, agarose (Sigma-Aldrich Solutions, Darmstadt, Germany), and 1x RedSafe. A 100 bp DNA ladder (Invitrogen, Karlsruhe, Germany) was used. The PCR product was mixed with 1 µL of dye, and 5 µL was loaded onto the gel. The sample was run at 100 Volt for approximately 30 minutes.

Table 4. Melting temperature of different flaviviruses generated during the development of the pan-flavivirus RT-qPCR protocol.

Virus Type	Melting Temperature (°C)
West Nile virus	79.0
Zika virus	81.0
DENV1	79.0
DENV2	81.0
DENV3	80.5
DENV4	80.5
JEV	81.5
Yellow fever virus	81.5
Negative control (primer)	74.5

The total RNA from the mosquito pool positive for the pan-flavivirus real-time PCR was sent for long non-coding RNA sequencing at Novogene, Hong Kong. The RNA library preparation included ribosomal RNA depletion as RNA enrichment, followed by paired-end sequencing with 150 base pairs (bp) per read using the Illumina NovaSeq 6000 sequencing platform, performed at Novogene. The number of raw reads reached around 50 million. The data analysis pipeline was used as described in a previous study [56]. Briefly, the raw clean reads were quality-trimmed using Trimmomatic v.0.36 (https://github.com/timflutre/trimmomatic (accessed on 26/09/2021)), followed by screening using Diamond v.0.9.15.116 (https://github.com/bbuchfink/diamond (accessed on 26/09/2021)) and BLAST v.2.6.0 against the complete NCBI non-redundant nucleotide and protein databases, with 1×10^{-5} as a cut-off e-value. Sequence reads that were indicated to match virus genomes were pulled out from the library by using seqtk/1.2 (https://github.com/lh3/seqtk (accessed on 26/09/2021)). These virus reads were then screened to extract the flavivirus sequences for phylogenetic inference. The reference genome of JEV was downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/ (accessed on 26/09/2021)) using "Japanese encephalitis virus" and "Vietnam" as search criteria. The dataset included 39 JEV sequences from Vietnam. All of the sequences were aligned using Multiple Alignment using Fast Fourier Transform (MAFFT) [57]. The phylogenetic analysis was performed using the IQ Tree web server, where a substitution model was selected first, followed by a pairwise maximum likelihood method for the tree inference [58]. Usutu virus (NC_006551) was included as an outgroup for the analysis.

In study II, 390 blood/serum samples of 197 patients (serum and blood samples collected at the same time point) were collected to identify the serotypes of the circulating DENV variants. The analysis was performed by using a pan-flavivirus RT-PCR assay targeting the flavivirus NS5 gene. The process of PCR running was conducted similarly as the *study I*. A sample was considered positive if it had a Ct-value below 35 with a correct melting temperature

(Table 4). The positive and suspected positive samples were sent for sequencing (Macrogen, Europe) for further confirmation and serotype identification.

Data analysis

In the first objective of study I, for the quantitative data analyses, Epidata (EpiData Association, Odense Denmark) was used for entry and data was transfer to STATA 15.0 (STATACorp Ltd, College station, Texas) for management and analysis.

In order to assess the knowledge of participants, their responses were marked by zero or one points in correspondence to the accurate and inaccurate answers to each question. The practice score was based on the sum of all methods that the participants have used to prevent and control MBDs. Higher scores were supposed to represent better levels of knowledge and practice. Chi-square, Fisher exact and Mann-Whitney tests were used to compare the differences between households with livestock and without livestock. Since the knowledge and practice scores were not distributed normally, Spearman's rho was used to describe the relationship between knowledge score and practice score. The Mann-Whitney test, the Kruskal Wallis test and negative binomial regression were used to identify factors associated with knowledge and practice scores. Factors with a p-value less than or equal to 0.1 in univariable analysis were included in the multivariable negative binomial model.

For qualitative data, all discussions were recorded and transcribed verbatim in word files. Content analysis was performed by organizing all interview texts into key themes. Only the quotations shown as illustrations for the findings were translated to English.

For data analyses of entomological data, Excel was used for data entry, and data were transferred to STATA 15.0, and merged with the questionnaire data for analysis. The Kolmogorov–Smirnov test was used to assess the normal distribution of variables. Spearman's rho was used to describe the correlation between the quantity of each of the types of mosquitoes and larvae with the numbers of pigs, cattle, and poultry kept. The number of mosquitoes collected was over-dispersed (variance was much higher than the mean), with excessive zero value; therefore, two zero-inflated negative binomial regression models was built to identify the factors associated with the size of the mosquito population. In the models, the total number of mosquitoes and the number of *Culex* mosquitoes were set as the outcome variables, since the other collected mosquito species were too few in quantity. Manual stepwise backward deletion of non-significant variables was applied to build the final models.

In study II, data was imported to Excel then transferred to SPSS and STATA for analysis. The associations between categorical variables were tested using Pearson's chi-square or Fisher's exact tests, while the mean of

continuous variables without normal distribution between groups were compared by Mann-Whitney test. Logistic regression analysis was used to identify the risk factors of dengue infection. Initially, all factors were tested in an univariable logistic regression model. The variables with p-values less than, or equal to 0.25 and suspected confounders and determinants found to be risk factors in the literature review were further analysed by multivariable analysis with dengue status as the dependent variable. Confounders were explored by comparing the difference between the adjusted odds ratio in multivariable analyses and the crude odds ratio in univariate analyses. In the next step, models were built by manual backward deletion of highly non-significant variables but keeping the KAP score variables as important predictors. Eventually, the determinants in the final model included gender, age, district where the patient lives, knowledge, attitude and practice scores. Odds ratios (OR) with 95 % confidence interval (CI) were reported to present the association. The analyses were also repeated with the scores calculated without weights, with each question contributing maximum 1 point, to see the impact on the final model. For the description of dengue patients' clinical symptoms and vital markers; mean, standard deviation, min, max values and percentage were utilized for descriptive statistical analysis. The association between categorical variables or categorical and continuous variables were tested by Pearson's chi-square or Fisher's exact or Mann-Whitney tests.

In both studies, Cronbach Alpha was used to test the internal consistency of the used questionnaire. A p-value less than 0.05 was considered statistically significant for all analyses.

Ethical statement

Ethical approval for *study I* was obtained from the Ethical Committee of Hanoi University of Public Health (No 406/2018/YTCC-HD3). The research ethic for *study II* was approved by the Ethical Committee of Bach Mai hospital No 690/QD-BM/2019.

Participation in both studies was voluntary. All human subjects were informed about the study purposes and gave verbal and written consent to participate. The research team committed that all information was handled anonymously and research data was only accessed by the research team.

Results

Knowledge and practice on MBDs prevention and control of citizen in the community

Our results indicated that the community members possessed basic knowledge on MBDs with an average score of 18.3 out of 35, of which non-livestock keeping households had a better knowledge than households keeping livestock (p<0.05). Both household categories had low scores, 3.5 out of 11, regarding preventive practices against MBDs.

Factors associated with knowledge and practice on MBDs

The negative binomial model showed that occupation and location of living were factors associated to the knowledge on MBDs. Compared to households in peri-urban districts, households living in central urban districts were more likely to have better knowledge regarding MBDs (p=0.016). Being farmers, unemployed or retired correlated to a poorer knowledge on MBDs as compared to office workers (p<0.05). Farmers were likely to have better preventive practices as compared to office workers (p<0.05). Those who had better knowledge also had more adequate preventive practices against MBDs (p<0.001).

Perspective on livestock keeping as a risk factor of MBDs

The qualitative data revealed that livestock keeping was determined as an increasing risk of MBDs from both health staff and community citizen's perspectives, due to an increase of mosquito populations. However, climatic variables or urbanization were considered more important.

> "It depends much on the weather. A lot of mosquitoes will appear in the humid weather after Tet holidays…Keeping livestock will have more mosquitoes

than no livestock keeping. Simple speaking, it's different between animal shelter area and space inside the house. There are less mosquitoes inside my house" - IDI with household with livestock in Dan Phuong district.

"It is certain that rearing pigs and chickens can increase the amounts of mosquitoes" - IDI with household with livestock in Chuong My district.

"Livestock attracts the mosquitoes because they serve as blood feeders. Mosquito will find them for their meals. Also, keeping livestock can leave the standing water that is the breeding sites for mosquitoes. But climatic factors are more important. Besides, our district has a fast speed of urbanization with many construction sites, many abandoned houses, unfinished construction works with many breeding sites for mosquitoes like underground water tanks…" - IDI with Ha Dong district health center's staff

"It is also a risk factor when people keep livestock nearby their home, especially in the past and in the mountainous areas. However, in my opinion, it's not the main factor but others like temperature, humidity…." - IDI with Hanoi Center of Disease Control's staff

The distribution and composition of collect vector population

In total, 12,861 mosquitoes and 2,427 larvae samples were collected. The distribution of the vector population in Hanoi city was heterogeneous.

Culex was the most dominant genus with a considerable percentage of more than 93% in total. *Cx. tritaeniorhynchus* was most common (67.18%), followed by *Cx. gelidus, Cx. quinquefasciatus* and *Cx. vishnui* with an approximately equal percentage of 8%. *Aedes* was least common with the proportion of around 0.7% only in five identified genera. *Aedes albopictus* was more common than *Ae. aegypti*. Other genera, including *Mansonia, Armigeres* and *Anopheles,* ranged from 1.21% to 3.82%. Mosquitoes were presented mostly in peripheral districts, while less mosquitoes were found in central districts, especially in Ba Dinh. Ha Dong had the highest number of mosquitoes collected, followed by the Dan Phuong district. *Culex tritaeniorhynchus* accounted for the highest proportion in peri-urban and peripheral districts while *Cx. quinquefasciatus* was the dominant species in the central districts. *Aedes aegypti* was found mainly in central urban districts whereas it could not be found in peripheral districts. In contrast, *Ae. albopictus* appeared in peri-urban and peripheral districts much more often than in central urban districts. In central urban districts, *Armigeres* and *Anopheles* were not found, while only one *Mansonia* mosquito was found there.

While more adult mosquitoes were trapped in peripheral districts, the number of larvae was much higher in peri-urban districts as compared to peripheral ones. The lowest numbers of both mosquitoes and larvae were however found in central urban districts. In contrast with the adult *Aedes* mosquitoes, their larvae accounted for the majority. *Ae. albopictus* were the most common larvae collected, with more than 80% of the total number of larvae collected,

while the percentage of *Ae. aegypti* larvae was 2.84%. Most of the *Ae. albopictus* larvae were collected in the peri-urban districts, followed by peripheral districts. *Ae. aegypti* larvae were mainly collected in central urban districts; however, their quantity was still lower than that of *Ae. albopictus* larvae. A total of 12.2% of the larvae collected belonged to the *Culex* species and were mostly found in the peripheral district of Ha Dong and in the peri-urban district of Chuong My. Notably, only one *Culex* larva was collected in Dan Phuong district, and no *Culex* larvae were detected in Bac Tu Liem district. There were 18 *Armigeres* larvae found in total, all from the peripheral district of Bac Tu Liem. Only one *Anopheles* larva was collected in Chuong My district.

The link between urban livestock and mosquitoes

Spearman's rho test showed that the number of pigs kept was positively correlated with the numbers of total mosquitoes, total mosquitoes indoors, total mosquitoes outdoors, total *Culex*, *Culex* indoors, *Culex* outdoors, total *Anopheles*, *Anopheles* indoors, *Anopheles* outdoors, total *Armigeres*, *Armigeres* outdoors, total *Mansonia*, *Mansonia* indoors, and *Mansonia* outdoors. No positive correlation was found between the numbers of mosquitoes and larvae and the numbers of cattle kept. While the test indicated a weak positive correlation between total *Anopheles*, *Anopheles* indoors, and *Anopheles* outdoors, all larval species were positively correlated with the quantity of poultry.

We built two zero-inflated negative binomial regression models using the Vuong test to identify the factors correlated with the quantity of mosquito population. Our results indicated that there was a positive association between pig rearing and the size of the mosquito population - particularly the *Culex* mosquito population. Households with better preventive practices reduced the possibility of mosquito presence. In particular, in both models, the factor of livestock keeping in the part of the logit model predicting excessive zeros was not statistically significant (p > 0.05). The expected log-change in the number of mosquitoes was 0.01 for a one-unit increase in pigs kept (p < 0.001) and −0.09 for one-unit increase in the practice scores of household respondents (p < 0.05). In the first model, the peri-urban districts had an expected log number of mosquitoes of 1.1 lower than peripheral districts and 1.5 higher than central urban districts (p < 0.001). In the second model, the peri-urban districts had an expected log number of mosquitoes of 1.2 lower than peripheral districts and 1.4 higher than central urban districts (p < 0.001).

Flavivirus presence in mosquito population in Hanoi

Out of 385 mosquito pools, only one pool of *Cx. tritaeniorhynchus* showed a suspected positive result (Table 5), with a Ct value of 38.27. This mosquito samples were from the Hong Phong commune, Chuong My district. From the sequencing results, we obtained 116,961,210 clean reads, with an efficiency of 97.82%, after removing the low-quality reads and adaptors. The paired-end sequencing read 1 and read 2 were combined, and the reads were mapped to the database (NCBI non-redundant nucleotide and protein database). Two reads matched to the polyprotein of JEV strain SXYC 1523 (GenBank accession No. ARX98191 for the amino acid and KY078829 for the nucleotide sequences, respectively). The 216 bp JEV sequence was extracted and used in a dataset to create a phylogenetic tree. The phylogenetic tree showed that the JEV sequence recovered from the mosquito pool belonged to genotype I (GT-I); however, the sequence was too short for further analysis.

Table 5 – Number of mosquito pools and a positive pool for flaviviruses by RT-PCR

Species	Number of pools	Positive pool
Cx. tritaeniorhynchus	248	1
Cx. vishnui and *pseudovishnui*	34	0
Cx. quinquefasciatus	34	0
Cx. gelidus	32	0
Cx. fuscocephala	1	0
Armigeres	6	0
Mansonia	7	0
Aedes	6	0

Risk factors of dengue infection and clinical characteristics of patients in the hospital

In the hospital-based study, the mean score of knowledge items and practice items of both dengue and non-dengue patients was 7.9 out of total 19 points and 3.9 out of total 17 points, respectively. The multivariable logistic regression indicated that older patients had lower risk of getting dengue infection as compared to younger adults aged 16-30, and patients living in peri-urban districts were less likely to suffer from DF than patients living in central urban districts (OR = 0.31; 95% CI 0.13-0.75). This study could not find any association with occupation, water storage habit, knowledge, attitude, or practice on dengue prevention. Among the dengue patients, the most prominent symptoms before admission were high fever above 39°C (80.61%), joint and muscle pains (43.88%) and headache (37.76%). Most patients with DF in the hospital started recuperating quickly after hospitalization.

Molecular epidemiology of dengue virus

Samples from 43 patients (33 dengue patients and 10 non-dengue patients) were suspected positive by RT-PCR. Interestingly, the majority of blood samples had lower Ct value than the serum samples, indicating that the titre of DENV in whole blood is commonly higher as compared to the serum (Figure 3). The sequencing data indicated that there were three serotypes of DENV co-circulating among the dengue patients including DENV-1, DENV-2, and DENV-4. Nineteen patients were infected by DENV-1, four patients by DENV-2 and only one patient was infected by DENV-4.
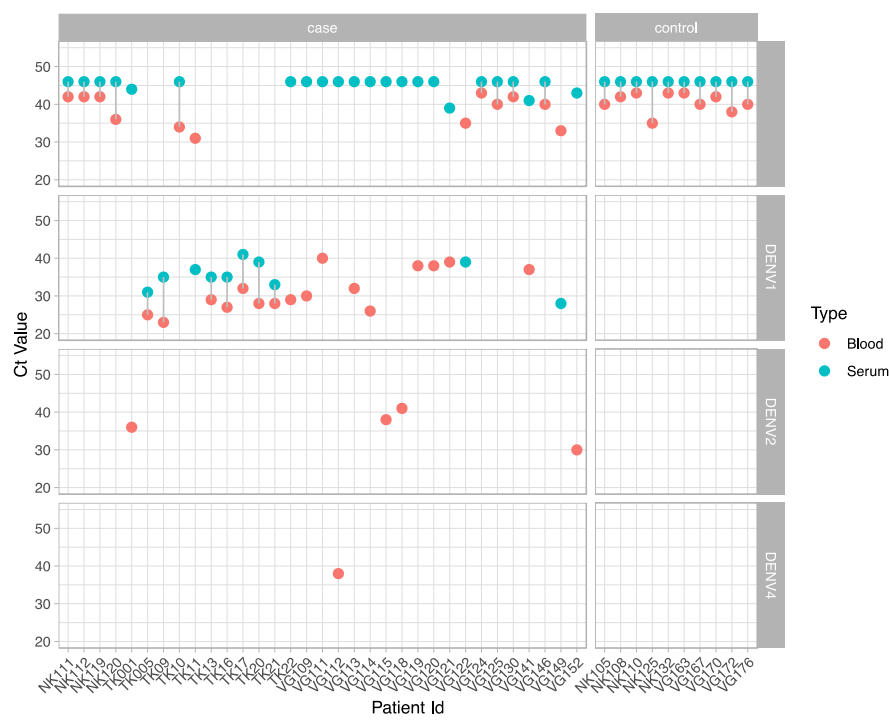
Figure 3 - Comparison of blood and serum samples from 33 dengue and 10 control cases, with positive or suspected positive dengue RT-PCR results

Discussion and future perspectives

This thesis sheds light on the urban epidemiology of mosquito-borne fla-viviruses and the role of livestock keeping in the transmission cycle in a large tropical city with a multi-pronged approach, including mixed methods to assess knowledge and practices on MBDs prevention and control, entomological collections of mosquitoes and larvae, molecular epidemiological methods, and a case-control study of patients to examine risk factors of DENV.

The main results of this thesis indicated that the district lived in, pig keeping, and preventive practices of people were associated with the abundance of mosquito populations. As compared to peri-urban districts, central urban districts had fewer mosquitoes, whereas the peripheral districts had much more mosquitoes. A possible explanation for this finding might be related to the activity of livestock keeping in general, and pig rearing in particular. Rearing of pigs and other livestock were common agricultural activities of all areas in Hanoi city in the past; however, with the rapid urbanization and industrialization of the capital city, this practice has recently been restricted in the central urban areas [59]. This may explain why fewer mosquitoes were found in these areas as compared to peripheral and peri-urban districts where livestock keeping is still common.

Environmental factors could also contribute to the differences in the presence of mosquitoes between the three categories of area. In our case, the domestic wastewater and sewer systems - which are important breeding sites of many mosquito species, such as *Aedes* [60,61] and *Culex* [62] - are more developed in the central urban districts as compared to the peri-urban districts. Meanwhile, peripheral districts are in a mixed situation, between central urban and peri-urban districts, with considerable speed of urbanization, while retaining the habit of keeping livestock. The development in these areas has created more breeding grounds for mosquitoes, such as stagnant water at construction sites, abandoned houses, or livestock-keeping activities amidst residential areas, leading to the increased possibility of mosquito population growth in these areas. In our study, a majority of the mosquitoes were collected in these peripheral districts, followed by the peri-urban districts. Our questionnaire survey's finding also showed that people living in central urban districts had better knowledge about MBDs than people living in peri-urban districts, and people with better knowledge about MBDs had better practices against

MBDs. This could also explain why we found fewer mosquitoes in the central urban districts than in the peri-urban districts.

Rearing of pigs can potentially provide blood meals for *Culex* mosquitoes, and pigs act as amplifying hosts for JEV [2]. Our study concluded that pig keeping increased the number of mosquitoes - especially *Culex* mosquitoes. This is further convincing evidence to support a previous study that was conducted in an urban area of southern Vietnam [43]; in that study, Lindahl and co-workers found that there was a strong association between rearing pigs and an increased presence of the *Cx. tritaeniorhynchus* population - the main vector of JEV. The laboratory analyses in this work detected flavivirus in one *Cx. tritaeniorhynchus* pool. Thereafter, it was attempted to recover the viral sequences from the initial RT-qPCR screening, but without success, probably because of the low copy number. By using RNA-Seq, two reads mapped to JEV genotype I were found, which was also consistent with our qPCR results. However, only two reads from over 100 million could be obtained, suggesting that the virus load in the *Cx. tritaeniorhynchus* mosquito pool was extremely low. Combining all results, this mosquito pool most likely carried JEV genotype I, which is one of the dominant genotypes in Asia [63–65]. Moreover, this result was expected, since this mosquito pool was from the Hong Phong commune, Chuong My district, where one human JEV case was recorded in 2019 (unpublished data). In one study conducted earlier in the Ha Tay province (now belonging to Hanoi city), JEV was isolated from two pools of *Cx. tritaeniorhynchus* [66]. Hence, our study shows an existing risk of JEV transmission in Hanoi city - especially in the peri-urban and peripheral districts, with the high abundance of the primary vector of JEV.

Cattle keeping was not correlated with the number of mosquitoes, although other studies suggest that cattle may contribute to higher numbers of *Anopheles* mosquitoes [41,67]. However, in some cases, the keeping of cattle, or ruminants such as goats and sheep, is used as a means of zooprophylaxis in some malaria-endemic countries [40]. Poultry keeping was correlated with the number of *Anopheles* mosquitoes and various kinds of larvae. To our knowledge, no evidence has been found to demonstrate the relationship between poultry keeping and the growth of any species of mosquito. *In study II*, I also strived to investigate whether livestock keeping was a risk factor for dengue infection. However, the number of individuals keeping livestock in this study was low. In contrast to JEV, dengue infection doesn't need any livestock in its transmission cycle, which could explain why no association between livestock keeping and dengue infection was found. Similarly, in our smaller study, we could also not find any association between livestock keeping including pig keeping, poultry-keeping, and the keeping of ruminants and the presence of *Aedes* mosquitoes [44]. As indicated in entomological data, *Aedes* mosquitoes were the least collected as compared to the other mosquito species; nevertheless, it is remarkable that their larvae - especially *Ae. Albopictus* - were more commonly detected than those of the other species. An aggressive spread of

Ae. aegypti and *Ae. albopictus* has been recorded across the globe [19,68]. These two *Aedes* species, which constitute the primary and secondary vectors of DF, were found in all districts. This finding implies that there is an existing risk for people living in both urban and suburban areas of Hanoi city to become infected with DENV. A previous study in the Khanh Hoa province, Vietnam, also proved that people in rural areas had at least an equal risk of getting DF compared to people in urban areas [69]. In fact, in our hospital-based study, the dengue patients came from all three categorized districts with the majority number living in central urban districts. This result could be explained by the entomological data regarding to the highest quantity of *Aedes aegypti* larvae collected in the central urban districts.

The study found that a better practice score reduced the number of mosquitoes. This finding was expected, as the abundance of mosquitoes will be decreased when people more often use preventive practices against mosquito growth. For instance, in a study conducted in Ho Chi Minh City, Vietnam, the results indicated that appropriate cover of containers diminished the risk of the presence of *Ae. aegypti* larvae as compared with those with inappropriate cover [70]. Similarly, people in suburban Washington who practiced source-reduction had lower numbers of *Ae. albopictus* and *Cx. pipiens* pupae in the containers [71]. Moreover, in Thailand, Rahman et al. [72] revealed that lack of dengue-preventive practices was significantly associated with higher abundance of adult female and immature *Ae. aegypti*. As shown in our studies, the preventive practices of households with and without livestock or patients in the hospital was not good, and people often felt powerless with regards to mosquito prevention or perceived it as somebody else's responsibility [73]. This could also be due to the low level of knowledge on MBDs prevention and control that was observed in both community-based and hospital-based studies.

Regarding the method used for mosquito collection, BG-Sentinel traps (with lures) were used indoors only, because they need to connect with a power source. This kind of trap is attractive for *Ae. aegypti*, *Ae. albopictus*, *Cx. quinquefasciatus*, and other selected species [74]. Non-baited CDC light traps were more flexible to use both indoors and outdoors, and can catch a wide range of mosquito genera, including *Culex* and *Aedes* species [75]. Therefore, we used more CDC light traps to collect mosquitoes for the entomological investigation. Other methods that could have been used to collect mosquitoes were backpack aspirators or gravid traps. We previously utilized backpack aspirators for collecting mosquitoes in the mentioned smaller study regarding livestock keeping and dengue. However, not many *Aedes* mosquitoes were collected, perhaps because this method was not optimal due to the time difference in collection between households over the course of a day [44]. Gravid traps were deemed to have a narrow spectrum in catching mosquitoes and could easily be in the way in the household areas. Hence, along

with the available logistic arrangement, we decided to use the combination of CDC light traps and BG-sentinel traps.

These studies have several limitations. Since participants' practice was only self-reported, information bias could happen. In addition, these studies focused only on Hanoi and may not be representative enough to extrapolate to other cities in Vietnam and other countries.

The findings from both studies suggest that vector control measures should be applied in all areas of Hanoi city, taking livestock keeping into account, in order to decrease the vector population and, thus, prevent the risk of transmission of flaviviruses such as DENV and JEV. Additionally, the work of my thesis leads me to recommend that community campaigns to raise the awareness and change the behavior on MBDs prevention and control should be organized. These should be done in collaboration between the health sector and the veterinary sector for households both with and without livestock living in central urban and peri-urban areas, especially for the pig farmers with regard to JEV prevention and control. Also, it is needed to increase the immunization rate of JEV vaccination (both primary and booster doses) - at least for the most vulnerable group of children under 15 years old in the endemic area. Further studies are needed to confirm the association between urban livestock keeping and potential increasing risks of MBDs in cities including DF and JE.

Conclusion

My studies revealed that there was a gap in community knowledge about MBDs prevention and control, although I could show that participants with better knowledge on MBDs generally had better practices against MBDs. However, the findings indicated a low level of preventive practices against MBDs of participants in both community-based and hospital-based studies. This was one of the risk factors causing the increase of vector population transmitting flavivirus-related diseases. Age was associated with the risk of dengue infection while location of living was an important factor leading to the abundance of mosquito populations and higher risk of getting MBDs. *Culex* genus was the most abundant mosquito found, and there was a positive association between pig rearing and the number of *Culex* species ($p < 0.001$). Hence, livestock keeping was determined as increasing risks of MBDs due to the growth of mosquito population. One pool of *Cx. tritaeniorhynchus*, collected in a peri-urban district, was found to be positive for JEV showing the presence of the virus.

Three dengue serotypes were confirmed to co-circulate in the same vector season, and *Aedes* larvae was found in large quantity compared to another genus, particularly in the city centre. This confirms that Hanoi city is an endemic area for MBDs such as JE and DF, and my work provides more evidence on the risk factors of flavivirus transmission in urban areas of Hanoi city related to the spread of *Culex* and *Aedes* mosquitoes.

In short, this thesis is increasing the knowledge regarding mosquitoes-borne flavivirus circulation from multiple perspectives, through community-based and hospital-based studies in Hanoi city, Vietnam where urban livestock keeping is considered as a risk factor of zoonotic transmission. The findings provide evidence to advise policymakers and stakeholders about the need of more effective prevention and control of MBDs transmission including DF and JE, and the risks of urban livestock keeping.

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