MINISTRY OF EDUCATION AND TRAINING
MINISTRY OF HEALTH
NATIONAL INSTITUTE OF HYGIENE AND EPIDEMIOLOGY

TRAN HAI SON

SPECIES COMPOSITION AND DISTRIBUTION OF SANDFLIES (DIPTERA: PSYCHODIDAE) AND THE CURRENT SITUATION OF FLAVIVIRUS AND LEISHMANIA INFECTION IN 6 PROVINCES OF NORTHERN VIETNAM

Specialization: Microbiology
Code: 62 42 01 07

SUMMARY OF DOCTORAL THESIS IN BIOLOGY

Hanoi – 2024

THIS THESIS WAS COMPLETED AT NATIONAL INSTITUTE OF HYGIENE AND EPIDEMIOLOGY

Science instructor:

1. Tran Vu Phong, PhD
2. Assoc. Prof. Nguyen Le Khanh Hang

Reviewer 1: Assoc.Prof. Vũ Đức Chính, PhD
Institute of malariology parasitology and entomology

Reviewer 2: Assoc.Prof. Đinh Đoàn Long, PhD
VNU - School of Medicine and Pharmacy

Reviewer 3: Huỳnh Hồng Quang, PhD
Institute of malariology parasitology and entomology - Quy Nhơn

The thesis will be defended at the National Institute of Hygiene and Epidemiology.
At the time ……., date …….., month ……, year 2024.

The thesis can be found at :

1. National Library
2. Library of the National Institute of Hygiene and Epidemiology

LIST OF PUBLISHED ARTICLES RELATED TO THE THESIS

1. Vu SN, Tran HS, Tran VP, Tran CT, Tran ND, Dang DA, Nguyen TY, Vu TL, Ngo KP, Nguyen VH, Hoàng NA, Cassan C, Prudhomme J, Depaquit J, Rahola N, Bañuls AL (2021), "Taxonomical insights and ecology of sandfly (Diptera, Psychodidae) species in six provinces of Northern Vietnam", Parasite. 2021;28:85. doi: 10.1051/parasite/2021080. Epub 2021 Dec 17. PMID: 34928207; PMCID: PMC8686828.
2 . Tran Hai Son, Nguyen Le Khanh Hang, Tran Vu Phong, Tran Cong Tu, Nguyen Viet Hoang, Vu Thi Lieu, Nguyen Thi Yen, Ung Thi Hong Trang, Vu Sinh Nam (2022), "The current status of Leishmania infection in populations of sandfly that was collected in 6 provinces of Northern Vietnam, 2016", Journal of Preventive Medicine, volume 32, number 8, 2022.

INTRODUCTION

Sandflies are arthropods belonging to the insect class, order Diptera, family Psychodidae and subfamily Phlebotominae. They feed on human and animal's blood and transmit pathogens such as viruses and parasites. They have been known as vectors of Leishmania and other pathogens in humans and animal. Sandflies are the main vector of disease caused by Leishmania, which is endemic in more than 98 countries with 350 million people at risk and over 2 million new cases every year.

Sandflies in transmitting Flaviviruses is unclear, although there is some evidence of Flaviviruses or Flavivirus RNA related to sandfly such as Saboya virus isolated from sandfly in Senegal (1991-1992), two Flavivirus sequences have been detected in the sandfly *Phlebotomus perniciosus* in Algeria (2007), Ecuador Paraiso Escondido virus (EPEV) in Ecuador (2011) or West Nile virus in Niger (2016). Flavivirus RNA has also been detected in Phlebotomine sandflies from Portugal. In 2014, Son La province recorded a large-scale outbreak of viral encephalitis lasting from June to September with 164 cases, of which 21 deaths. In recent years, mountainous areas such as Hat Lot and Son La have also continuously recorded Dengue outbreaks ranging from several dozen to several hundred cases.

We conducted the study "Species composition and distribution of Sandflies (diptera: psychodidae) and the current situation of Flavivirus and Leishmania infection in 6 provinces of Northern Vietnam" with 3 **objectives**:

1) Determine the species composition and some distribution characteristics of sandflies in 6 provinces of Northern Vietnam, 2016-2018.
2) Describe the current status of Flavivirus infection in sandflies in this study
3) Describe the current situation of Leishmania infection in sandflies in this study

New scientific points and practical value of the topic :

Scientific significance, novelty, and timeliness of the problems studies

The thesis has contributed some new scientific points and practical value of the topic:

1. New scientific points:

- This is a new study that covers many contents related to the epidemiological characteristics of the distribution of sand mosquitoes and plants infected with infectious pathogens in Vietnam. The study contributes epidemiological distribution data of 5 genera and 13 species of sand mosquitoes and 2 unidentified species, their role as disease vectors;
- Determining evidence of female sand mosquitoes carrying Flavivirus agents and protozoan parasites Leishmania spp. causes disease in humans, opening up future research directions, helping epidemiologists, entomologists, biologists & molecular biologists and infectious disease clinicians to further recognize the causes of emerging infectious diseases in Vietnam. , especially when the earth's temperature gradually warms up - good weather conditions for sand mosquito populations to increase due to shortening the development cycle;
- Although sand mosquitoes usually live in caves and rarely in residential areas, there is now evidence of their presence outside caves and livestock cages in endemic areas, so this may be a new issue that needs attention. health aspects of ecotourism, caves and in these sand mosquito endemic areas.

2. Scientificity

- The thesis applies classical research methods and techniques of morphology, molecular biology, and gene sequencing to identify the breed and species of sand mosquitoes as well as the pathogens Flavivirus and Leishmania spp. in the northern provinces of Vietnam;
- The research process involved cooperation between the National Institute of Hygiene and Epidemiology and research partners in Montpellier, France, so the results were highly accurate and reliable.

3. Practicality

- The research results in the thesis provide new data, warning of emerging infectious diseases in Vietnam, opening up directions for future in-depth research on both epidemiology, microbiology, clinical practice, treatment and prevention. prevention measures;
- Research data can serve as reference material, clinical practice, and undergraduate and graduate teaching in microbiology, parasitology,

and entomology.

Structure of the thesis

The thesis includes: 117 pages excluding references and appendices, 12 tables, 39 figures and 1 diagram. Question 2 pages. Overview 45 pages; Research objects and methods 20 pages; Results 26 pages; Discussion 20 pages; Conclusion 2 pages; Recommendations 1 page; Research limitations 1 page.

Chapter 1. OVERVIEW

1.1. Sandfly and some epidemiological characteristics

Of the more than 800 recognized sandfly species, approximately 464 species was found in the New World and 375 species in the Old World. Phylum: Arthropoda, Class: Insecta, Order: Diptera, Suborder Nematocera, Family: Psychodidae, Subfamily: Phlebotominae (Bigot 1854, K.KertÉsz 1903). The Phlebotominae subfamily is divided into six genera: three from the Old World (Phlebotomus [13 subgenera], Sergentomyia [10 subgenera], and Chinius [4 species]) and three from the New World (Lutzomyia [26 subgenera and group], Brumptomyia [24 species] and Warileya [6 species]). Currently, 78 species of Sandflies have been proven to be vectors of Leishmaniasis. Among the sandfly vectors mentioned above, 7 species are involved in the transmission of *L. major* , 7 species transmit *L. tropica* , 31 species transmit *L. infantum* , and 9 species transmit *L. donovani* .

Sandflies are insects with an complete metamorphosis life cycle. In the development cycle there are 4 distinct phases: egg, larva, pupa and adult. In Vietnam, sandflies are quite common and can be found in many different habitats. Sandfly have been recognized as vectors of Leishmaniasis transmission in Vietnam since the 1930s.

1.2. Flavivirus and some epidemiological characteristics

1.2.1. General characteristics of Flavivirus

1.2.1.1. Classification

The family Flaviviridae includes 4 genera: Flavivirus, Hepacivius, Pestivirus and Pegivirus. Genus Flavivirus has more than 53 members including vector-borne diseases caused by Dengue virus causing Dengue fever, Dengue hemorrhagic fever, Dengue shock syndrome; Japanese encephalitis virus; Yellow fever virus causes yellow fever; Chikungunya virus, Kyasanur Forest disease, Murray Valley

encephalitis, Omsk hemorrhagic fever, tick-borne encephalitis, West Nile fever, and Zika.

1.2.1.2. Morphological characteristics and genetic material structure of Flavivirus group

Viruses of the Flavivirus group are spherical, 40 - 60 nm in diameter, inside the core of the virus is the nucleocapsid which is the structure of the virus genome and protein C. The nucleocapsid is surrounded by a membrane (viral shell) which is a lipid double layer, contains glycoproteins and proteins derived from the cell's plasma membrane.

The flavivirus genome is a positive-strand RNA with a CAP structure at the 5' end and specifically lacks a poly-A tail at the 3′ end [55]. The viral genome encodes a single polyprotein that, after transcription by viral and host proteases, form 10 structural proteins (C-prM-E-NS1-NS2A-NS2B-NS3-NS4A- NS4B-NS5)

1.2.2. Virus replication

Flaviviruses replicate in the cytoplasm and virus particle assembly occurs in intracellular vesicles.

1.2.3. Antigenic properties

Flaviviruses all share the same antigenic site. At least eight antigenic complexes have been identified based on neutralization experiments.

1.2.4. Laboratory diagnostics

1.2.4.1. Detection of virus RNA

The reaction performed to detect Flavivirus is RT-PCR or Realtime RT-PCR, in which Realtime RT-PCR gives results faster than traditional RT-PCR. The RT-PCR reactions and processes currently used for detection focus on the target gene segment encoding the virus envelope (E-encoding gene), encoding the envelope membrane (M/E-encoding gene), encoding (pE) and encode proteins NS5, NS3, NS1.

1.2.4.2. Virus isolation method

The virus isolation method usually takes 1-3 weeks, so it does not meet the requirements for quick diagnosis, but the results obtained by this method provide a lot of virus information for virus research. biology, pathology and vaccine development

1.2.4.3. Detection of anti-antibodies

Serological methods will be complicated in places where many viruses belonging to the Flavivirus group (DENV, Japanese B encephalitis,

yellow fever) circulate.

1.2.5. Diseases caused by Flaviviruses transmitted by vectors are circulating in Vietnam

Diseases caused by Flaviviruses transmitted by vectors circulating in Vietnam include Dengue, JE, and Zika.

1.3. Leishmania and some epidemiological characteristics

1.3.1. Taxonomic rank of Leishmania

Kingdom	Protista (Haeckel, 1866),
Class	Kinetoplastea (Honigberg, 1963 emend. Vickerman, 1976),
Subclass	Metakinetoplastina (Vickerman, 2004),
Order	Trypanosomatida (Kent, 1880),
Family	Trypanosomatidae (Döflein, 1901),
Subfamily	Leishmaniinae (Maslov and Lukeš 2012) (Ross, 1903).

1.3.2. Leishmania parasite and life cycle

There are about 21 species of the genus Leishmania that cause disease in humans . They can be distinguished on the basis of biological criteria, or laboratory analysis (mainly isoenzyme analysis and DNA analysis), or different clinical and epidemiological symptoms.

1.3.3. Genome characteristics of Leishmania

Leishmania has unique genome organization features compared to eukaryotes, such as intronless genes, polycistrons, and small chromosomes with high gene density. Furthermore, these flagellates possess a single mitochondria called the kinetoplast, which contains a large network of kinetoplast DNA (kDNA).

1.3.3.1. Chromosomal DNA

Ribosomal RNA (rRNA) genes are located mostly on chromosome 27, often existing in multiple copies with a size of approximately 12.5 kb. Among the various components of these genes, the ITS regions are ideal for species identification. The 18S rRNA is a structural RNA of the ribosomal SSU. The high conservation of this gene and its flanking regions makes it suitable for reconstructing phylogenetic relationships.

1.3.3.2. Genes encode proteins

Members of leishmania possess 36 chromosomes, except for the *L. mexicana complex* which has 34 chromosomes. The Viannia genus has

35 chromosomes. The Sauro Leishmania subgenus has 38 chromosomes. The Leishmania genome is compact with a size of 33 Mb.

1.3.3.3. Extra-chromosomal DNA

All kinetoplastid flagellates possess a unique mitochondrial genome called kDNA, which consists of several thousand circular DNA molecules linked together in an interconnected network of thousands of minicircles (approx. 1 kb per ring) and several dozen maxicircles (approximately 23 kb per ring).

1.3.4. Method for diagnosing Leishmaniasis in the laboratory

1.3.4.1. Method for determining Leishmaniasis

Different diagnostic methods and the possibility of detecting, identifying and quantifying Leishmania species, as well as their ability to differentiate at different levels (genus, subgenus, species, species complex, species and population)

1.3.4.2. Method for distinguishing Leishmania species complexes

Identification and differentiation of leishmania species complexes and species can be accomplished through various molecular biological techniques.

Nested PCR and semi-nested PCR can be used to distinguish species with appropriate primers.

The Sanger method is rapidly improving in quality, read length, speed, and cost, and it is widely used for identification of leishmania species complexes and phylogenetic studies.

Next generation sequencing (NGS) technology in the analysis of the Leishmania genome has recently facilitated the discovery of various genetic diversity including single nucleotide polymorphisms (SNPs), variants copy numbers (CNVs), structural variations in detail and provide valuable insights into the complexity of the genome and gene regulation. Genome analysis of Leishmania presents a challenge because of the frequent presence of aneuploidy. This compromises the accuracy of detecting all genetic variations.

1.3.5. Leishmaniasis and some epidemiological characteristics

1.3.5.1. Epidemiology of Leishmaniasis in the world

Leishmaniasis affects mostly poor people in developing countries; 350 million people are considered at risk of Leishmaniasis, and about 2

million new cases occur every year. Approximately 95% of CL cases occur in the Americas, Mediterranean basin, Middle East and Central Asia with 132,568 cases reported in this regions in 2012. In its report on the health burden of infectious and parasitic diseases worldwide, Hotez ranked Leishmaniasis 9th with 2,357,000 cases annually, mainly occurring in Africa, Southeast Asia, and East Asia. Mediterranean, Western Pacific, America and Europe, of which Southeast Asia is the hardest hit with about 67.3% of the total number of cases worldwide.

1.3.5.2. Epidemiology of Leishmaniasis in Vietnam

From 1978 to 2018, cases of the disease were reported sporadically, mainly in the Northern region of Vietnam. A total of 6 patients were reported to be related to the leishmaniasis parasite, including 4 cases with confirmed co-infection with HIV.

1.3.6. Clinical features, treatment and prevention

According to clinical characteristics, the disease can be divided into the following 3 types:

1.3.6.1. CL-cutaneous Leishmaniasis

Leishmaniasis CL resides in the skin, with an incubation period of several weeks to several months. The causative agent is infection with *L. major* or *L. tropica*

1.3.6.2. Visceral Leishmaniasis (VL- Visceral Leishmaniasis)

Visceral Leishmaniasis resides in internal organs, the incubation period in most cases is 3-6 months, in some cases several weeks to several years. The main symptoms are fever, spleen swelling, increased blood Iggamma, acute anemia, and leukopenia. Without treatment, the patient will die within two years from complications of wasting and secondary infection.

1.3.6.3. Mucocutaneous Leishmaniasis (MCL)

Leishmaniasis of the skin and mucous membranes is less common.

1.3.6.4. Treatment and prevention

Treatment of VL is usually performed with agents belonging to the group of pentavalent antimonials (meglumin antimonate, sodium stibogluconate) pentamidine, or amphotericin B.

Chapter 2 . MATERIALS AND METHODS

2.1. Content 1

Objective 1: Determine the species composition and some distribution characteristics of sandflies in 6 provinces of Northern Vietnam, 2016-2018.

2.1.1. Study population: Sandflies were collected from six selected provinces: Quang Ninh, Ninh Binh, Lang Son, Lao Cai, Ha Giang and Son La from May 30, 2016 to October 13, 2016

2.1.2. Study design: Cross-sectional descriptive survey

2.1.3. Sample size: A total sandflies were collected in 6 provinces of Northern Vietnam

2.1.4. Sampling and laboratory methods

Collecting sandflies in different habitats: Using CDC miniature light traps (CDC miniature light traps, John W. Hock Co. FL, USA) to collected sandflies; screening sandflies in the field: Distinguishing sandflies from other types of mosquitoes based on typical morphological characteristics. Male sandflies and female sandflies were stored in 1.5ml tubes containing 70% alcohol and in liquid nitrogen, respectively.

Sandfly identification based on the identification key of Lewis (1978, 1987) and Killick Kendrick et al. (1991) adds comparisons with the descriptions of Newstead (1911), Raynal (1936), Abonnenc E. 1972, Johnson H. 1991 and Lewis 1982 [11, 25, 181-184]. Sample images were observed with the camera system on a nilkon E600 electron microscope and analyzed with NIS-Elements software. The images of species identification results were sent and confirmed at the Montpellier Research Institute, France (IRD).

2.1.5. Variables

Abundance: RA = (Total number of species/Total number of individuals) x 100

Density: D = Total number of individuals/Total number of traps set/Number of nights of setting traps

Significance level: Mean = Total number of individuals collected/Total number of traps set

Number of species: SR = Number of species in the collected habitat (including *Se. sp2* and *Se. sp3*)

2.1.6. Data analysis

Data were entered using Excel and analyzed using Stata ver 14 and Excel software. Images were taken and measured using NIS-Elements software. Use Kruskal–Wallis test statistical analysis to compare the distribution of Sandflies by province and habitat.

2.2. Content 2:

Objective 2: Describe the current status of flavivirus infection in sandflies in this study

2.2.1. Study object: Viruses in the genus Flavivirus, family Flaviviridae in specimens collected at objective 1.

2.2.2. Study design: Cross-sectional description with laboratory analysis

2.2.3. Sample size: Total 1009 thorax and abdomen samples of female sandflies were collected in objective 1

2.2.4. *Sampling and laboratory methods*

Flaviviruses identification by RT-PCR as Mirsada Hukić et all (2020). Samples positive for the Flavivirus will be subjected to Sanger sequencing. PCR product was purified using ExoSAP-IT™ PCR Product Cleanup Reagent, cycle sequencing using Big Dye Terminator 3.1. Sequencing PCR products were purified using the Dye Ex 2.0 Spin kit and put in the ABI 3100-AvantTM Genetic Analyzer

2.2.5. Data analysis

Data were entered using Excel and analyzed using Stata ver 14 and Excel software. Gene sequence analysis using the BLAST function on NCBI, identification on the Web Flavivirus Genotyping Tool Version 0.0.

2.3. Content 3

Objective 3: Describe the current situation of Leishmania in sandflies in this study

2.3.1. Research location: Department of Entomology and Animal Medicine - Department of Virology - National Institute of Hygiene and Epidemiology

2.3.2. Research object: Leishmania parasites on female Sandflies collected in target 1.

2.3.3. Study design: Cross-sectional description with laboratory analysis

2.3.4. Sample size: All thorax and abdomen samples of female Sandflies were collected: 1009 samples.

2.3.5. Biological products and equipment

- DNA/RNA extraction biological products: similar to section 2.2.5
- Nested PCR biological products: GoTaq; Primer pair for Nested-PCR reaction

Standard certification:

+ Positive control (POS): *Leishmania infantum* 680 bp.
+ Negative control (No Template Control - NTC): use water that does not contain DNase-RNase to check the process of mixing chemical products
+ Negative Extraction Control (NEC): use water that does not contain Dnase-Rnase to check the extraction process

- Equipment: similar to section 2.2.5.

2.3.6. Nested PCR reaction

Prepare biological products for Nested-PCR reaction: according to instructions of Gotaq biological kit (Promega), outer primer pair CBS2XF-CBS1XR, internal primer pair LIR-13Z

Judging results: Results are accepted when:

+ Positive control: has a specific band equivalent to the size of the designed primer pair of 680 bp.
+ Negative reaction control (NTC), negative extraction control (NEC): negative.

- Negative for leishmania group: appearance of PCR products in non-specific positions or no presence of PCR products.
- Positive for Leishmania group: specific PCR product with size over 500 bp. Corresponding band size: *L. amazonensis* MHOM/BR/73/LV78 (517bp); *L. major* MHOM/ET/95/FV1 (560-570 bp); *L. infantum* (680 bp); *L. tropica* (750 bp) [130].

- Samples positive by Nested-PCR are subjected to genetic sequencing (NGS).

2.3.7. Next generation sequencing (NGS) gene sequencing method

Use biological products and chemicals from the Nextera XT DNA Library Prep kit. Equalize the sample library with the ISEQ 100 machine using the Standard Normalization - Illumina method.

2.3.8. Blood meal analysis

Sample size: DNA from female sandflies recorded as blood-fed and positive for Leishmania will be used to determine blood meals. This is to determine whether this Leishmania strain related to the sandfly's blood meal is human or animal blood. However, in this study, we did not conduct blood meal analysis because the samples positive for Leishmaniasis in the study did not record blood.

2.3.9. Data analysis

Data were entered using Excel and analyzed using Stata ver 14 and Excel software. Data after sequencing, use FastQ file for analysis.

Chapter 3. RESULTS

3.1. Species composition and some distribution characteristics of sandflies in 6 provinces of Northern Vietnam, 2016-2018

3.1.1. Sandfly species composition by breed, density and abundance

Table 3.1. Number, sex, density and abundance of sandflies by species in 6 northern mountainous provinces of Vietnam, 2016-2018

Species	Number	Female/Male (*n)	Density	Relative abundance
Sergentomyia (*Neophlebotomus*) *sylvatica*	249	87/161(*1)	0.0253	9.632
Sergentomyia (*Parrotomyia*) *brevicaulis* group	66	49/17	0.0067	2.553
Sergentomyia (*Parrotomyia*) *barraudi* group	324	303/21	0.0329	12.534
Sergentomyia (*Sergentomyia*) *bailyi*	55	44/11	0.0056	2.128
Sergentomyia (*Neophlebotomus*) *hivernus*	49	46/3	0.0050	1.896
Sergentomyia (*Neophlebotomus*) *perturbans*	11	5/6	0.0011	0.426
Sergentomyia (*Neophlebotomus*) *khawi*	25	7/18	0.0025	0.967
Sergentomyia sp2	201	140/58(*3)	0.0204	7.776
Sergentomyia sp3	10	8/2	0.0010	0.387
Sergentomyia und_sp	83	65/18	0.0084	3.211
Sergentomyia (*Neophlebotomus*) sp.	4	4/0	0.0004	0.155
Sergentomyia sp.	990	50/928(*12)	0.1006	38.298
Grassomyia indica	6	1/5	0.0006	0.232
Phlebotomus (*Anaphlebotomus*) *stantoni*	102	46/55(*1)	0.0104	3.946
Phlebotomus (*Euphlebotomus*) *yunshengensis*	87	28/59	0.0088	3.366
Phlebotomus (*Larroussius*) *betisi*	50	3/47	0.0051	1.934
Phlebotomus (*Euphlebotomus*) *mascomai*	35	17/18	0.0036	1.354
Phlebotomus (*Euphlebotomus*) sp.	21	5/16	0.0021	0.812
Phlebotomus (*Larroussius*) sp.	12	4/8	0.0012	0.464
Phlebotomus sp.	33	25/8	0.0034	1.277
Idiophlebotomus sp.	14	6/8	0.0014	0.542
Chinius junlianensis	31	27/4	0.0031	1.199
NA	127	79/40(*8)	0.0129	4.913
Total	2585	1049/1511(*25)	0.2626	100

*#Number of specimens for which the gender could not be determined, NA: Not Available.

The results showed that there are 5 genera of sandflies: Sergentomyia (n=2067, 79.96%) accounts for the highest proportion, followed by Phlebotomus (n=340, 13.15%), Chinus (n=31, 1.2%), Idiophlebotomus (n=14, 0.54%) and Grassomyia (n=6, 0.23%) (Table 3.1).

A total of 13 species have been identified, of which Sergentomyia has 7 species; Phlebotomus species has 4 species and Chinius has 1 species.

3.1.2. Distribution of sandflies by province

The Sergentomyia is the most dominant in the 6 provinces. Statistical analysis shows that the distribution by breed between provinces is not significantly different. But the species distribution, including *Se. sp2* and *Se. sp3* , is very different between provinces (p-value =0.002, α=0.05).

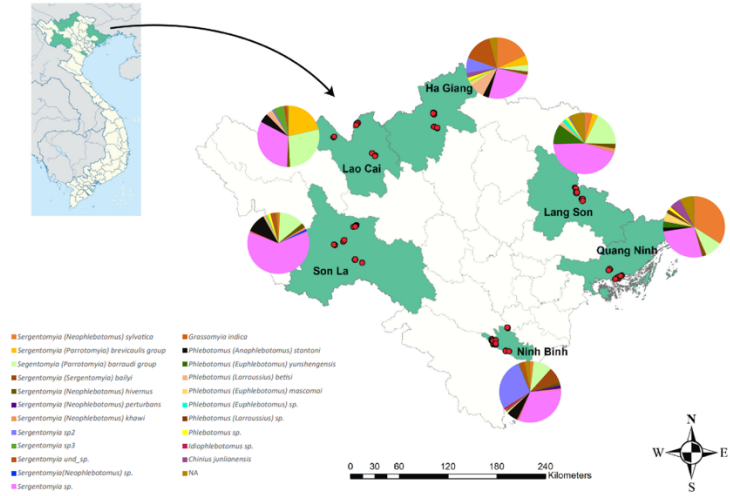


Figure 3.1. Collection points and composition of sandfly species in 6 provinces of Northern Vietnam, 2016

3.1.3. Distribution of sandflies according to habitat

Sandflies were most commonly collected in caves (n = 1431, relative abundance RA = 55.36 and $_{\text{cave}}D = 0.79$). The highest species richness was in caves ($_{\text{cave}}SR = 15$, including *Se. sp2* and *Se. sp3*). Regarding relative abundance, we also collected many outdoor sandflies, with 936 specimens corresponding to RA=36.21 and species richness SR=15 (including Se. *sp2 and Se. sp3*). However, the density of sandflies collected by traps placed in dog cages was higher than that placed outside the house ($D_{\text{cages). dog}} = 0.36$, $D_{\text{outside the house}} = 0.23$). The density of sandflies indoors is low and similar to the density in chicken/poultry/duck coops and lower than the density in buffalo/cow/goat barns ($_{\text{Indoor}}D = 0.08$; $D_{\text{poultry barn}} = 0.10$; $D_{\text{barn}} = 0.12$). This distribution according to habitat is not significantly different among the 6 provinces. In caves, the number of species is the highest, all varieties and species have been found (Table 3.3 and figure 3.1). Statistical analyzes showed that the distribution of species is different depending on habitat (p-value <0.01, $\alpha = 0.01$).

Table 3.3. Number of sandflies, abundance, density and number of species according to habitat

Species	Buffalo/cow/ goat shed	Cave	Chicken/bird/ duck shed	Dog shed	Indoor area	Outdoor area (garden)	Pig shed
Sergentomyia (*Neophlebotomus*) *sylvatica*	1	141	14	1		92	
Sergentomyia (*Parrotomyia*) *brevicaulis* group		45				21	
Sergentomyia (*Parrotomyia*) *barraudi* group	4	172	4		1	138	5
Sergentomyia (*Sergentomyia*) *bailyi*	15	10	5		3	17	5
Sergentomyia (*Neophlebotomus*) *hivernus*	3	10	5	1		28	2
Sergentomyia (*Neophlebotomus*) *perturbans*		6			1	4	
Sergentomyia (*Neophlebotomus*) *khawi*	3	16			1	5	
Sergentomyia sp2	5	135	2		3	55	1
Sergentomyia sp3		9				1	
Sergentomyia und_sp.		20	1		2	60	
Sergentomyia (*Neophlebotomus*) sp.		4					
Sergentomyia sp.	12	566	12	2	6	378	14
Grassomyia indica		4				2	
Phlebotomus (*Anaphlebotomus*) *stantoni*	11	32	8	7	11	24	9
Phlebotomus (*Euphlebotomus*) *yunshengensis*		80		1		6	
Phlebotomus (*Larroussius*) *betisi*		19	1			30	
Phlebotomus (*Euphlebotomus*) *mascomai*	2	24		1		8	
Phlebotomus (*Euphlebotomus*) sp.		14	2			4	1
Phlebotomus (*Larroussius*) sp.		10	1	1			
Phlebotomus sp.	3	15	2	1		11	1
Idiophlebotomus sp.		7				7	
Chinius junlianensis	1	24				6	
NA	5	68	9	1	2	39	3
Total	65	1431	66	16	30	936	41
Relative abundance	2.51	55.36	2.55	0.62	1.16	36.21	1.59
Density	0.12	0.79	0.10	0.36	0.08	0.23	0.07
Mean (SF nb/CDC nb)	1.44	15.66	1.57	1.78	1.07	5.29	1.14
Species Richness*	9	15	7	5	6	15	5

3.1.4. Distribution of female sandflies in 6 mountainous provinces in Northern Vietnam, 2016

Female sandflies were collected in 6/6 provinces in the study. The highest number of female sandflies was in Ninh Binh and Lang Son with 298 individuals (28.41%) and 294 individuals (28.03%), followed by Ha Giang with 159 individuals (15, 16%), Quang Ninh 125 individuals (11.92%), Son La with 98 individuals (9.34%) and Lao Cai 75 individuals (7.15%).

3.2. Flavivirus infection in sandflies at the study site

3.2.1. Detection of Flaviviruses in female sandflies by RT-PCR

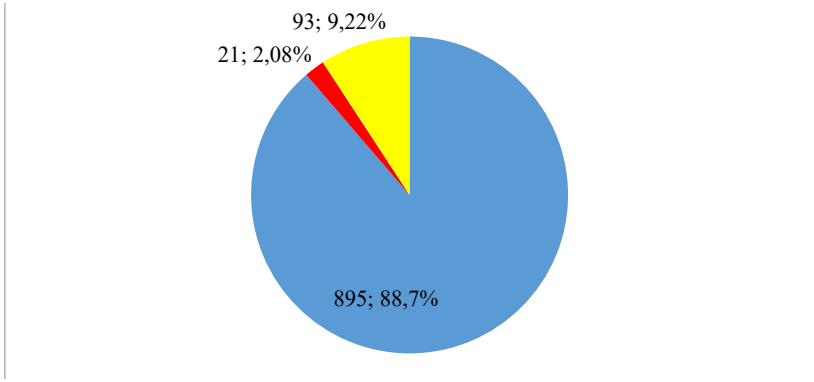
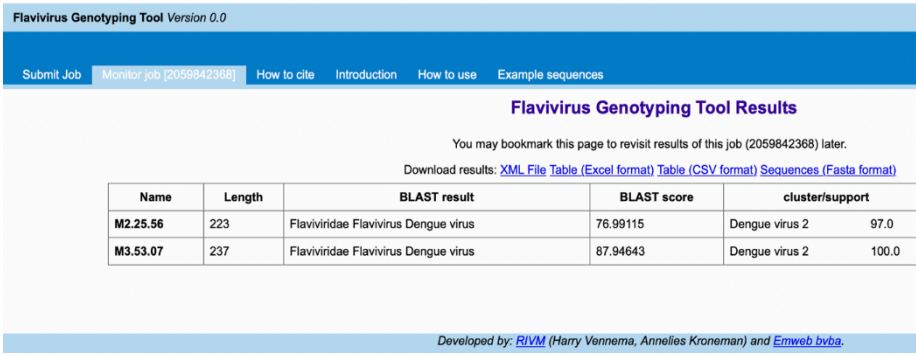


Figure 3.4. Results of Flavivirus screening on female sandflies

Results of Flavivirus screening on sandflies showed that 21/1009 samples had Flavivirus RNA related to sandflies (2.08%, red), 895 samples were determined to be negative for Flavivirus, accounting for 88.7% (blue), the remaining 93 samples had PCR products, but were not specific to flavivirus (9.22%, yellow) (Figure 3.4). All 21 samples positive for Flavivirus by RT-PCR will be subjected to gene sequencing to confirm the presence of Flavivirus in the collected sandfly population.

3.2.2. Identification of Flaviviruses by Sanger sequencing method

Among 21 samples, 16 samples did not find any information when comparing the sequences of those samples to the genetic database of NBCI. There were 3 samples with sequences showing similarities to Flavivirus, but the length of the homologous segment is too short (<31bp) to determine. There were 2 samples number 4 (M2.25.56) and 17 (M3.57.07) whose sequences were determined to be related to Dengue type 2 (DEN2).



3.2.3. Flavivirus characteristics in female sandflies

Among the 6 investigated provinces, screening results recorded the appearance of ARN fragments of Flaviviruses in general and DEN2 in female sandflies in 2 provinces, Ninh Binh and Lang Son. Ha Giang, Lao Cai and Quang Ninh, Son La have not found traces of Flavivirus in

sandfly populations. The proportion of female sandflies carrying ARN of Flavivirus (DEN2) in our study is 0.198% (n=2/1009).

Table 3.4. Flavivirus information on female sandfly populations

Provinces	Latitude	Longitude	Sandfly	Environment	Flavivirus
Ninh Binh (M2.25.56)	20°13.983'	105°42.704'	*Sergentomyia sp2*	Cave	DEN2
Lang Sơn (M3.53.07)	21°56.069'	106°41.061'	*Sergentomyia barraudi*	Cave	DEN2

3.3. Current status of Leishmania in sandflies

3.3.1. Detection of Leishmania using Nested-PCR

Of the 1009 samples screened, 20 samples were suspected of being infected with leishmaniasis and 989 samples were negative.

Table 3.6. 20 samples suspected of being infected with Leishman by Nested-PCR method

No.	Number	Code sample	Length product (bp)
1	02	Sample 68 (Pic 3.6-L1) Sample 895 (Pic 3.6-L6)	300
2	06	Sample 79 (Pic 3.6-L4) Sample 99 (Pic 3.6-L2) Sample 906, 907, 909, 910 (Pic 3.6-L7)	500
3	05	Sample 96 (hình 3.6-L1) Sample 181 (Pic 3.6-L5) Sample 900, 897 (Pic 3.6-L6) and 912 (Pic 3.6-L7)	600-750
4	07	Sample 69 (Pic 3.6-L2), Sample 75, 92 (Pic 3.6-L3) Sample 904, 905, 899 (Pic 3.6-L6) và 663 (Pic 3.6– L8)	>=800

All 20 samples will be genetically sequenced to confirm the presence of Leishmania in the collected sandfly population.

3.3.2. Identification of Leishmania species by NGS method

Gene sequencing using NGS method with Nextera XT DNA Library Prep kit, equalize the sample library with the ISEQ 100 machine using the Standard Normalization - Illumina method.

The results obtained after sequencing 20 suspect samples resulted in a total of 121,651 sequence fragments (10GB) with sizes ranging from 20 bp to 1728 bp (average 73,945 bp). We removed sequences that were too small in size (under 200bp) to obtain 71 gene sequences for analysis.

Among 71 sequences, 40 sequences had no information found when blasted to NBCI's gene database, 21 sequences showed homology but the species information obtained was not related to Leishmania. There are only 10 sequences identified as Leishmania belonging to 3 samples: 2, 4, 20. Samples 2 (Vietnam/NB28062006) and 20 (Vietnam/SL10102016) have gene sequences homology to classes 13, 16, 18, 25, 31, 38, 39 and 50 on the minicircle kinetoplas gene of *Leishmania infantum*, while sample number 4 (Vietnam/QN01062006) has a sequence homology to chromosome 27 of Leishmania, from position 251340 to position 268948.

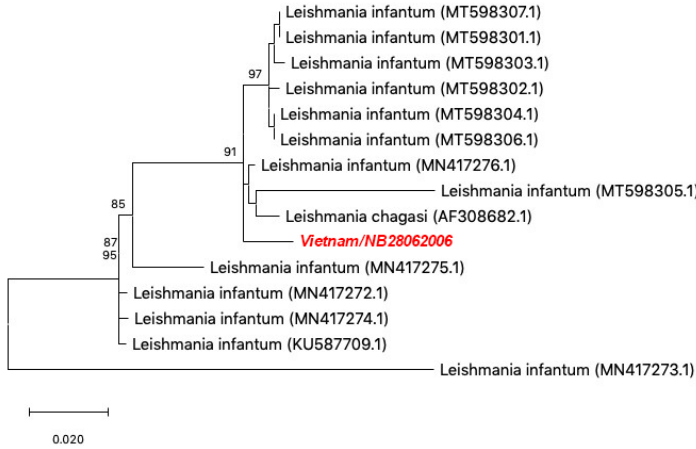


Figure 3.7. Phylogenetic tree of Leishmania in the female sandfly population in Vietnam (Maximum likelihood method, kDNA genes)

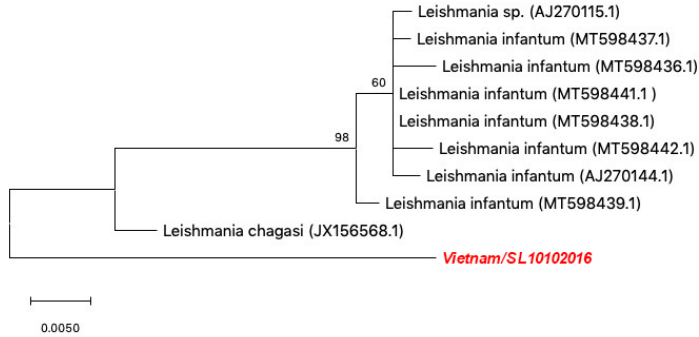


Figure 3.8. Phylogenetic tree of Leishmania in the female sandfly population in Vietnam (Maximum likelihood method, kDNA genes)

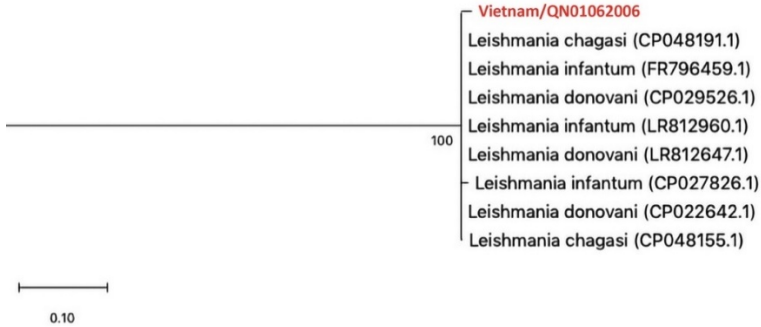


Figure 3.9. Phylogenetic tree of Leishmania in the female sandfly population in Vietnam (Maximum likelihood method, genes on chromosome 27)

Thus, the gene sequencing results showed that of the 3 samples with homology to Leishmania, 2 samples were identified as *Leishmania infantum* and 1 sample was identified as *Leishmania sp.*

3.3.3. Some characteristics of Leishmania in sandfly populations

Among the 6 provinces investigated, screening results recorded the presence of leishmaniasis in 3 provinces: Son La, Quang Ninh and Ninh Binh. The remaining provinces of Ha Giang, Lao Cai and Lang Son have not found traces of Leishmania in sandfly populations. In our

study, the rate of leishmaniasis infection in the female sandfly population was 0.297% (n=3/1009).

Table 3.9. Leishmania information on female sandfly populations

Provinces	Latitude	Longitude	Sandfly	Environment	Leishmania
Quang Ninh	20°59.615’	107°12.592’	*NA*	Cave	*Leishmania sp.*
Ninh Binh	20°14.457’	105°41.371’	*Sergentomyia sp2*	Outdoor	*Leishmania infantum*
Son La	21°11.063’	104°03.277’	*Phlebotomus sp.*	Pig shed	*Leishmania infantum*

Chapter 4. DISCUSSION

4.1. Species composition and some biological characteristics of sandflies in 6 mountainous provinces in Northern Vietnam, 2016-2018

4.1.1. Identification of sandfly species in Vietnam by morphological characteristics

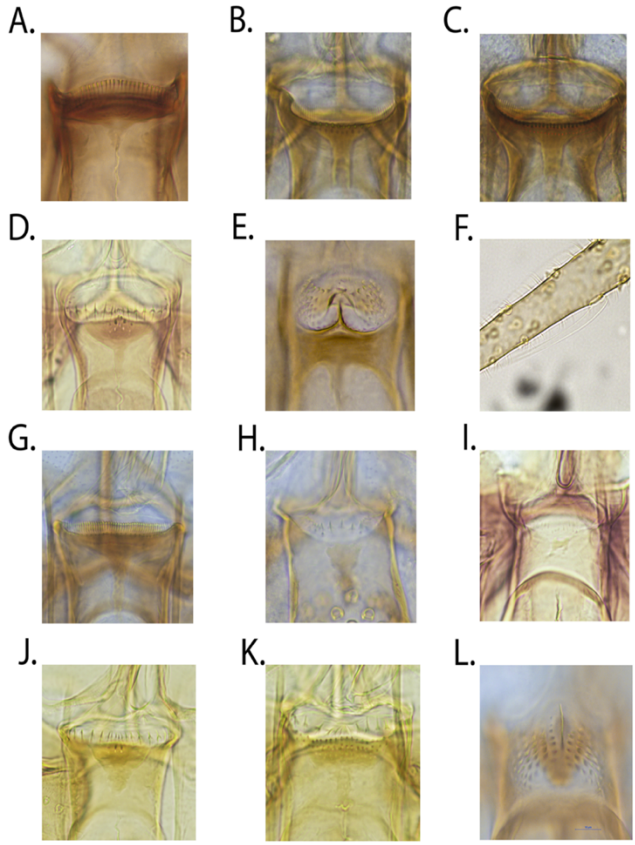


Figure 4.3. Cibarium and ascoid morphology of females

Grassomyia indica (A), *Sergentomyia barraudi group* (B, C), *Se. khawi* (D), *and Se. anodontis group (E), ascoid on Antennae 3 of Se. sp.3* (F) *, Se. brevicaulis group* (G), *Se. sylvatica* (H) *, Se. bailyi* (I), cibarium *of Se. hivernus* (J), cibarium *of Se. perturbans group* (K), *cibarium of Idiophlebotomus sp.* (L).

4.1.2. Ecology and habitat of sandflies in the investigated provinces

The highest species richness values were found in caves and outdoors (SR=15 including *Se. sp2* and *Se. sp3*). In barns and homes, species richness ranged from 5 to 9, suggesting more anthropophilic behavior or attraction to domestic animals for some species such as *Ph. stantoni*. In fact, although this species was found in all environments, it was the main species collected mainly in homes (11/30 specimens; 36.67%) and in dog houses (7/16 samples; 43.75%). In caves, this species accounts for only 2.24% (32/1431 specimens). Further studies on the

feeding preferences of this species are needed as it has been described as a cave species in Thailand (SRhang=26) and Malaysia (SRhang=18, n= 1548 **).**

4.2. Current status of Flavivirus infection in sandflies at the research site

4.2.1. Identification of Flavivirus in sandflies

In our study, the products was amplified by the RT-PCR with the cFD2/MAMD primers (250 bp) was sequenced to confirm the gene fragment.

The results of the study identified two sequences similar to the DEN2 virus. The difference in the study is that the sample we screened was the mash of female sandflies, while in Scaramozzino's study human samples were used. This shows that the DEN2 blister strains carried by female sandflies are related to the DEN2 patterns in patients. Although this does not prove the role of sandflies in transmitting DEN2 in particular and flavivirus in general, however, through this study we also see that it is entirely possible for sandflies to carry flaviviruses and especially DEN2. In the context that natural ecological zones are shrinking due to urbanization impacts, it is possible for viruses to also mutate to adapt to new conditions. In the future, we hope to have further studies on the disease transmission role of these virus strains.

4.2.2. Prevalence of Flavivirus infection in sandflies

Another study by Gregory Moureau in 2010 also announced the discovery of Flavivirus RNA in sandflies. In this study, 1508 sandflies collected in France and Algeria, from August 2006 to July 2007, 2 pools of males out of 67 pools of this species *Phlebotomus perniciosus* in Algeria were positive for Flavivirus. The results of these two pools have sequences similar to Flavivirus, related to insect vectors of the genus Culex. This is the first description of insect-specific flaviviruses of the Culicidae family (including Aedes, Culex, Mansonia, Anopheles mosquito genera), found in sandflies of the Psychodidae family. The difference between this study and ours is the discovery of Flaviviruses in male sandflies. Therefore, in the future, when screening for Flaviviruses on sandflies, we will recommend

conducting both on males and females to better understand the nature of Flavivirus viruses.

4.2.3. Some characteristics of Flaviviruses in female sandflies

Our detection of DEN2 traces on female sandflies of the genus Sergentomyia in Vietnam is not enough to confirm whether they transmit this virus or not. However, in the future, the genus Sergentomyia in general and the two species *Segentomyia (Parrotomyia) barraudi group* and *Segentomyia sp2* need to be studied further to understand their role in transmitting Flaviviruses, especially DEN2.

4.3. Current status of Leishmaniasis in sandflies and the risk of transmission to humans

4.3.1. Leishmania identified in Quang Ninh

Visceral leishmaniasis in humans was first reported in 2000 in Quang Ninh province. These cases raise questions about local transmission of leishmaniasis in Vietnam. Samples collected from a patient in Quang Ninh were identified by the Queensland International Institute, Brisbane, Australia as *Leishmania infantum* or *L. donovani*.

In this study, we screened 1 sample of *Leishmania sp.* from a sandfly caught in a cave near the patient's home in 2000. Genetic sequencing results of the Leishmania strain in the study determined that the sequence was 99.89% similar to *L. infantum* or *L. donovani* (Figure 3.9). This is completely similar to the results isolated from patients of the Queensland Inter-National Institute, Brisbane, Australia.

4.3.2. Leishmania identified in Son La

In the positive leishmania sample from Son La, we found 7 gene sequences similar to 8 classes (class 13, 16, 18, 25, 31, 38, 39 and 50) on the minicircle kinetoplas of the published *Leishmania infantum* strains. This shows the complexity of the genetic structure of this group of parasites. The circular genes on kinetoplasms are highly repetitive and multi-layered. Without using the NSG technique, it is very difficult to analyze and separate these repeats (Table 3.9).

4.3.3. Leishmania identified in Ninh Binh

Similar to the strain in Son La, the Leishmania sample we collected in Ninh Binh showed two sequences similar to the sequences

of *Leishmania infantum* strains published on class 16 and 25 of the minicircle kinetoplas.

Although these data are not enough to conclude *Se. sp2* is the vector that transmits *L. infantum*, but this discovery confirms for the first time the presence of Leishmania in the sandfly population in Vietnam, and is a strain that causes a very dangerous visceral disease. Sandflies of the genus Sergentomyia have never been recorded to transmit Leishmaniasis, so knowledge about them is still limited. With the results of the study, we think the role of disease vectors of the Sergentomyia genus should be increased and should be included in surveillance to clarify the disease transmission role of the Sergentomyia genus in general and the *Se. sp2* in particular

CONCLUSION

1. Species composition and some distribution characteristics of sandflies in 6 provinces of Northern Vietnam, 2016-2018

In 6 mountainous provinces in Northern Vietnam, 2585 sandflies were collected, including 1511 males (58.5%) and 1049 females (40.6%), recording 5 sandfly breeds: Sergentomyia (n=2067, 79.96%) accounted for the highest proportion, Phlebotomus (n=340, 13.15%), Chinus (n=31, 1.2%), Idiophlebotomus (n=14, 0.54%) and Grassomyia (n=6, 0.23%).

A total of 15 sandfly species have been recorded, of which 13 sandfly species have been identified, 2 sandfly species have been unidentified: *Sergentomyia sp2. and Sergentomyia sp3.*

Sandflies were recorded to be present in all habitats including: indoors (n=30), outdoors (n=936), bufalo cages (n=65), chicken cages (n=66), pigs cages (n=41), dog cages (n=16) and caves (n=1431). Sandflies are mainly collected in caves. The highest species richness was in caves and outdoor habitats SR=15 (including *Se. sp2 and Se. sp3*).

2. Current status of Flavivirus infection in sandflies at the research site

Proportion of female sandflies carrying RNA of DEN2 virus in the study was 0.198% (n=2).

3. Current status of Leishmaniasis in sandflies at the research site

Leishmania is determined on female sandfly populations in three provinces: Son La, Quang Ninh and Ninh Binh. Leishmania infection rate in female sandfly population was 3/1009 individuals (0.297%).

RECOMMENDATION

1. Increase surveys to collect sandflies in other provinces to have more data to compare with the results of the study.
2. Continue and expand studies on morphology and molecular biology to accurately identify mosquito vectors that transmit diseases and further clarify the role of pathogens such as Flaviviruses, especially DEN2 and leishmaniasis on sandflies for a comprehensive picture.
3. Expand research on the behavior and ecology of sandflies in habitats near humans to have proactive prevention measures in the context of finding DEN2 and Leishmania RNA in female sandflies.

RESEARCH LIMITATIONS

Research has discovered two new sand mosquito species: *Se. sp2* and *Se. sp3* but the species has not yet been identified. Therefore, it is time to continue to collect more related information, and coordinate with domestic and foreign sand mosquito experts to confirm the species.

The finding of evidence of Leishmania spp. on the female sand mosquito population in Vietnam is a very new discovery. However, the role of transmitting the protozoan parasite Leishmania spp has not been clarified. of sand mosquitoes in Vietnam, additional evidence is needed such as the ability to transmit and multiply the virus in the bodies of those sand mosquitoes. In the future, when it is possible to raise and propagate sand mosquitoes and isolate Leishmania spp. disease, will help provide more complete information about the disease transmission role of this vector in Vietnam.

Comparison with other studies that have detected Flavivirus in sand mosquitoes through isolation of virus strains, allowing to obtain more complete sequences of virus gene segments. In this study, the results were not isolated so the results were limited to recording evidence of Flavivirus in female sand mosquito samples collected.

On the other hand, studies around the world show that male sand mosquitoes are also capable of being infected with Flavivirus. In this study, screening was performed on female sand mosquitoes. Therefore, in the future we will conduct research on male sand mosquito samples.