![Aix*Marseille université - Socialement engagée]

THÈSE DE DOCTORAT

Soutenue à Aix-Marseille Université

Le 25 Novembre 2022

Ly Na HUYNH

MALDI-TOF MS and Molecular Tools for the Identification of Arthropods of Medical and Veterinary Importance in Vietnam

Discipline
Biologie et santé

Spécialité
Maladies infectieuses

École doctorale
ED 62 : Sciences de la vie et de la santé

Laboratoire/Partenaires de recherche
Vecteurs-Infections Tropicales et Méditerranéennes (VITROME)

Institut Hospitalo-Univisitaire (IHU) Méditerranéennes

Composition du jury

Pr. Jérôme DEPAQUIT   Rapporteur
Faculté de Pharmacie
Université de Reims Champagne Ardenne

Pr. Anna COHUET   Rapporteuse
IRD, Montpellier
Unité Maladies infectieuses et vecteurs : écologie, génétique, évolution et contrôle

Pr. Arezki IZRI   Président de jury
Service de Parasitologie-Mycologie
AP-HP, HUPSSD, Hôpital Avicenne, Université Paris 13

Pr. Philippe PAROLA   Directeur de these
Aix-Marseille université
Institut Hospitalo-Universitaire

![MÉDITERRANÉE INFECTION logo] ![VITROME logo]

Affidavit

I, undersigned, Ly Na HUYNH, hereby declare that the work presented in this manuscript is my own work, carried out under the scientific direction of Pr. Philippe PAROLA, in accordance with the principles of honesty, integrity and responsibility inherent to the research mission. The research work and the writing of this manuscript have been carried out in compliance with both the French national charter for Research Integrity and the Aix-Marseille University charter on the fight against plagiarism.

This work has not been submitted previously either in this country or in another country in the same or in a similar version to any other examination body.

Place Marseille, date 24 August 2022

Ly Na HUYNH

List of publications and participation conferences

1. List of the pulication produced as part of this thesis project

Article 1: MALDI-TOF mass spectrometry identifcation of mosquitoes collected in Vietnam

Ly Na Huynh *et al*., 2022. **Published in** Parasites & Vectors, **PMID:** 34582467

Article 2: Morphological, molecular and MALDI-TOF MS identification of ticks and tick-associated pathogens in Vietnam

Ly Na Huynh *et al*., 2021. **Published in** PLoS Negl Trop Dis, **PMID:** 34582467

Article 3: Morphological, molecular and MALDI-TOF MS identification of fleas species and their associated microorganisms in Vietnam

Ly Na Huynh et al., 2022. **Targeted journal:** Microorganisms

Article 4: Mosquitoes and Mosquito-borne diseases

Ly Na Huynh et al., 2022. **Submitted in** Insects

2. Participation in conferences during this thesis period

Poster: Proteomics tool for the identification of medical arthropod in human and veterinary importance in Vietnam

Infectiopôle sud day, **2020-** IHU méditerannée infection, Marseille, France

Poster: Morphological, molecular and MALDI-TOF MS identification of ticks and tick-associated pathogens in Vietnam

Infectiopôle sud day, **2022-** IHU méditerannée infection, Marseille, France

ACKNOWLEDGMENTS

DEDICATION

I dedicate this PhD thesis to:

*A special feeling of gratitude to my loving parents **Huynh Tan Luc** and **Pham Thi Hien** for their unconditional, loving support, and encouragement in keeping me confident and having peace of mind. This thesis is also dedicated to my father-in-law **Ngo Tung Chau** without his endless love, understanding, and encouragement I would never have been able to complete my studies. I am grateful for everything that all of you have done for me.*

*I deepest thank **my younger sister's family**, who take care of my little daughter, My thanks to little niece **Nguyen Quynh Anh** for always being a close friend to **Bap** during my absence from home to study abroad.*

*Feeling gratitude to my beloved husband **Ngo Tung Quan** for his faithful, awaiting, support, and love. I am the luckiest woman in the world to have you in my life.*

*To my little daughter, **Ngo Tue Kim** for her strong, awaiting, and obedience to grandparents during my absence from home. You are always in my heart bebe.*

TABLE OF CONTENTS

Summary

Medically important arthropods include insects and arachnids are vectors of disease and can transmit many infectious pathogens (bacteria, protozoa, and viruses) that cause common and emerging diseases between humans or from other creatures to humans. Annually, transmitted infectious agents are responsible for millions of infectious and death cases in humans and animals around the world. Integrated vector management and epidemiological surveillance are essential in strategies for the control and monitoring of these vector-borne diseases. Therefore, the accurate identification of arthropod vectors and the characterization of the repertoire of associated microorganisms are crucial steps in vector control. This study aimed to determine arthropods captured from wild and domestic mammals in Vietnam and the presence, prevalence of their associated microorganisms using morphological, molecular, and MALDI-TOF approaches.

In the first project, we evaluated the capacity of the MALDI-TOF MS tool for the rapid and correct identification of the field-caught Vietnamese mosquitoes stored in silica gel. The MALDI-TOF MS analysis allowed us to identify 22 mosquito species, of which 18 species, were added to our house database to be reference spectra. Our work indicated that the MALDI-TOF MS was able to distinguish between the sibling mosquito species and cryptic species groups that are not only difficult to distinguish morphologically but also molecularly.

In the second project of the thesis, we combined three tools (morphology, molecular, and MALDI-TOF MS) to identify alcohol-preserved ticks and to search for microorganisms associated with these ticks collected in Vietnam. In this work, we showed the usefulness of MALDI-TOF MS to overcome the limitations of traditional morphological and molecular

identification methods. We also explored for the first time, the DNA of five microorganisms (*Anaplasma platys*, *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Ehrlichia rustica*, and *Theileria sinensis*) was detected in dogs and cattle ticks.

The third project allowed us to identify two flea species that were stored in alcohol using the MALDI-TOF MS technique. The effectiveness of the approach to distinguish between species in the same group was evaluated as well. Fleas were also screened using molecular biology tools to detect microorganisms associated with their ectoparasites. We reported for the first time in Vietnam the presence of *Bartonella* genus (*B. clarridgeiae*, *B. rochalimae*, *B. coopersplainsensis*) and *Wolbachia* endosymbiont in fleas collected in wild small rodents and domestic animals.

Finally, we wrote a literature review of mosquitoes and mosquito-borne diseases in Vietnam. Our review was to provide a complete checklist of all Vietnamese mosquitoes that have been recognised, along with a comprehensive and systematic overview of mosquito-borne diseases in Vietnam. This review may be useful to decision-makers responsible for vector control strategies and to researchers for future surveys on mosquitoes.

Overall, our thesis enabled us to confirm the effectiveness of MALDI-TOF MS in medical entomology, for the first time its application on Vietnamese mosquitoes, ticks, and fleas collected in the field in the central highlands of Vietnam, and to detect the important vector-borne agents and their associated diseases.

Résumé

Les arthropodes d'importance médicale et vétérinaire sont des insectes et des arachnides pouvant transmettre de nombreux agents pathogènes (bactéries, protozoaires, et virus) responsables de maladies chez les Humains ou les animaux. Chaque année dans le monde , ces agents infectieux transmis sont responsables de millions de cas infectieux, certains mortels, chez les humains et les animaux . La lutte intégrée contre les vecteurs et la surveillance épidémiologique sont essentielles dans les stratégies de contrôle et de surveillance de ces maladies à transmission vectorielle. Cette étude visait à identifier les arthropodes capturés sur des mammifères sauvages et domestiques au Vietnam et analyser la présence éventuellede micro-organismes pathogènes à l'aide d'études morphologiques, de la biologie moléculaires et de la technique du MALDI-TOF.

Dans le premier projet, nous avons vérifié la capacité de l'outil MALDI-TOF MS pour l'identification des moustiques vietnamiens capturés sur le terrain et stockés avec du gel de silice. L'analyse MALDI-TOF MS nous a permis d'identifier 22 espèces de moustiques, dont 18 espèces, ont été ajoutées à la base de données IHU comme spectres de référence. Nos travaux montrent que le MALDI-TOF MS est capable de faire la distinction entre des espèces de moustiques très proches ainsi que pour des groupes d'espèces cryptiques, difficiles à distinguer morphologiquement mais aussi par biologie moléculaire.

Dans le second projet de la thèse, nous avons combiné trois outils (morphologie, biologie moléculaire, et MALDI-TOF MS) pour identifier des tiques conservées dans l'alcool et pour rechercher les micro-organismes associés à ces tiques collectés au Vietnam. Dans ce travail, nous avons montré l'utilité du MALDI-TOF MS pour aller au delà des limites des méthodes traditionnelles d'identification morphologique et moléculaire. Nous avons également exploré

pour la première fois l'ADN de cinq micro-organismes -*Anaplasma platys*, *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Ehrlichia rustica*, et *Theileria sinensis* - détectée chez des chiens et des tiques de bovins.

Le troisième projet nous a permis d'identifier à l'aide de la technique MALDI-TOF MS deux espèces de puces stockées en alcool. L'efficacité de la techniquepour distinguer des espèces d'un même groupe a également été évaluée. Les puces ont été criblées à l'aide d'outils de biologie moléculaire pour détecter les micro-organismes associés. Nous avons ainsi signalé pour la première fois au Vietnam la présence du genre *Bartonella* (*B. clarridgeiae*, *B. rochalimae*, *B. coopersplainsensis*) et de l'endosymbionte *Wolbachia* dans des puces collectées chez des petits rongeurs sauvages et des animaux domestiques.

Enfin, nous avons rédigé une revue de la littérature sur les moustiques et les maladies transmises par les moustiques au Vietnam. Le but était de fournir une liste tous les moustiques vietnamiens connus, ainsi qu'un aperçu complet des pathogènes transmis par ces moustiques. Cette revue peut être utile aux décideurs responsables des stratégies de lutte antivectorielle et aux chercheurs pour de futures enquêtes sur les moustiques.

Globalement, notre thèse nous a permis de confirmer l'efficacité du MALDI-TOF MS en entomologie médicale. Il a été utilisé pour la première fois sur les moustiques, tiques et puces collectés sur le terrain dans les hauts plateaux du centre du Vietnam, et identifier les vecteurs importants et les maladies associées.

Introduction

Medical entomology is focused on the study of insects and other arthropods that have a huge impact on human health. A "One Health" perspective emphasizes the interdependence of human, veterinary health, and their ecosystem. Community awareness of vector biology, arthropod monitoring, and vector population control will continue to be important, as will emergency measures to prevent the spread of vector-borne diseases. Thus, medical entomology research and monitoring are critical in the fight against arthropod-borne diseases (1).

Arthropods are the most diverse phylum, accounting for more than 85% of all known living animal species (2). Hematophagous arthropod vectors consume a pathogen agent (bacterium, parasite, or virus) while blood-feeding on an infected vertebrate host before transmitting the pathogen to another (3). Mosquitoes and ticks have been identified as the first and second most important vectors of human and animal infectious pathogens, respectively, that are responsible for hundreds of thousands of human infections and deaths each year, globally (1). Many emerging and re-emerging infectious diseases have been transmitted to humans and animals for decades by blood-feeding arthropods such as mosquitoes, ticks, fleas, lice, and other blood-sucking arthropods (4). Many different factors contribute to their emergence and re-emergence, including microbial evolution, population growth, reservoir hosts or vectors, human behavior, and ecosystem imbalance factors, posing a threat to human and animal health (5).

For effective arthropod control, accurate identification of arthropod vectors from non-vectors is essential. Morphological identification is the "gold standard" method, which is widely used for discriminating between arthropods using their external characteristics. However, this traditional method has several drawbacks, including the requirement for standard taxonomic keys, expert

5

entomology knowledge, and the ability to classify species belonging to siblings, cryptic groups, and immature or damaged specimens (6,7). Molecular biology techniques such as polymerase chain reaction (PCR), enzyme electrophoresis, and DNA sequencing have been used to distinguish between homogeneous species (8). However, these techniques are also limited because they are time-consuming, expensive, and require primer-specific targeting for certain species (9,10).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has revolutionised the field of clinical microbiology and mycology (11). Recently, MALDI-TOF MS has been proposed as an alternative and innovative method of overcoming the drawbacks of the above two methods in medical entomology to identify arthropods (10). This approach has routinely been applied in our laboratory for the rapid and accurate identification of arthropods, including mosquitoes, ticks, lice, bedbugs, and termites (12–15). MALDI-TOF MS is an easy-to-use, time-saving, and affordable method compared to earlier methods, and a device may be purchased for medical microbiology and then used in entomology (16). Its operation is based on the identification of acidic extraction of proteins from an organ of interest. The mass/charge ratio of each protein is measured as it passes across an electric field after propulsion of the protein molecules from ultraviolet laser desorption. The mass/charge ratio of the proteins generated is a unique protein mass spectrum of the specific sample known as the "protein signature" for reliable species identification. This spectrum is then compared to a database of reference profiles containing the spectra of species that have been officially validated using morphology and molecular techniques (10).

Vietnam is a tropical country in Asia where individuals are at high risk of acquiring arthropod-borne diseases such as malaria, dengue, Zika, Japanese encephalitis, murine typhus, and scrub typhus (17,18). In which, malaria and dengue fever are the most prevalent diseases and continue

to be public health threats. As a result, the Vietnamese government is heavily investing in malaria elimination to achieve great success by 2030 through a variety of programs and strategies such as social-economic improvement, early diagnosis, prompt and effective treatment, widespread distribution of insecticide-treated bed nets, and regular use of pyrethroids through indoor residual spraying in households (18–21) as well as reduce and prevent dengue by improving knowledge and behavior changes, vector management (22). Additionally, many studies have focused on malaria and dengue and their agents that are transmitted by mosquitoes, but much less on other arthropods. For instance, the most common microorganisms are transmitted by fleas and ticks such as cat-scratch disease is transmitted by cat fleas *Ctenocephalides* spp. (agents *Bartonella henselae* and *Bartonella clarridgeiae*), *Pulex irritans* flea was described as *Bartonella* vector, which can cause trench fever (23), feline spotted-fever-causing bacteria (*Rickettsia felis*), *Rhipicephalus* (*Boophilus*) *microplus* tick as a vector of *Anaplasma phagocytophilum* that is the causative agent of human granulocytic anaplasmosis and tick-borne fever in ruminants (24), and Scrub typhus is caused by *Orientia tsutsugamushi* bacterium (formerly *Rickettsia tsutsugamushi*) and is transmitted to humans via the bite of trombiculid mite larvae (25). The emerging and reemerging infectious diseases are caused by ectoparasites is significant increasing, however, limited data is available on ectoparasites and ectoparasite-borne microorganisms in Vietnam. Therefore, the aim of this thesis was to use MALDI-TOF MS tool to identify arthropods from Vietnam and to detect microorganisms associated with their arthoropods. We hope that this thesis may be useful to entomologists conducting future surveys of Vietnamese arthropods and their related microorganisms as well as decision-makers responsible for vector control tactics.

The result of our research is presented in the following published or submitted papers: In the first project, we assessed the ability of the MALDI-TOF MS tool for the rapid and correct identification

of the field-caught Vietnamese mosquitoes stored in silica gel. The MALDI-TOF MS analysis allowed us to identify 22 mosquito species, 18 of which were added to our house database as reference spectra. Our work indicated that the MALDI-TOF MS was able to identify sibling mosquito species and cryptic species groups that are difficult to distinguish not only morphologically but also molecularly (Article 1). In the thesis's second project, we used three tools (morphology, molecular, and MALDI-TOF MS) to identify alcohol-preserved ticks and look for microorganisms associated with ticks collected in Vietnam. In this study, we demonstrated the usefulness of MALDI-TOF MS in overcoming the limitations of traditional morphological and molecular identification methods. For the first time, we investigated the DNA of five microorganisms (*Anaplasma platys*, *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Ehrlichia rustica*, and *Theileria sinensis*) found in dogs and cattle ticks (Article 2). The MALDI-TOF MS technique was used in the third project to identify two flea species that were stored in alcohol. The approach's ability to distinguish between species in the same group was also evaluated. Fleas were also screened for microorganisms associated with their ectoparasites using molecular biology tools. The presence of the *Bartonella* genus (*B. clarridgeiae*, *B. rochalimae*, *B. coopersplainsensis*) and *Wolbachia* endosymbiont in fleas collected from wild small rodents and domestic animals was reported for the first time in Vietnam (Article 3). Finally, we completed this project by conducting a literature review on mosquitoes and mosquito-borne diseases in Vietnam. Our goal was to provide a complete checklist of all recognized Vietnamese mosquitos, with a total of 281 mosquito species of 42 subgenera and 22 genera found in Vietnam, as well as a comprehensive and systematic overview of mosquito-borne diseases in Vietnam (Article 4).

ARTICLE I

MALDI-TOF mass spectrometry identification of mosquitoes collected in Vietnam

Ly Na Huynh, Adama Zan Diarra, Hong Sang Nguyen, Long Bien Tran, Van Nguyen Do, Van Hoang Ho, Xuan Quang Nguyen, Philippe Parola*

Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a tool that has revolutionised clinical microbiology and has recently been described as an innovative and effective approach to arthropod identification. In this study, mosquitos were captured in Vietnam using four different methods: human landing catch (HLC), CDC light traps (CDC-LT), BG sentinel traps, and animal-baited net traps. A total of 4,215 mosquitoes were captured and morphologically identified as belonging to three genera: *Aedes*, *Anopheles,* and *Culex*. These species are widely distributed, medically important mosquitoes, and were most frequently captured during entomological surveillance and management programmes in the Central Highlands of Vietnam. We randomly selected 1,253 mosquitoes, including 662 specimens of 14 *Anopheles* species, 200 specimens of two *Aedes* species, and 391 morphologically unidentified *Culex* specimens for molecular and MALDI-TOF MS analysis. For *Culex* mosquitoes that were not identified to the species level when specific morphological characters could not be identified with certitude due to being damaged during collection, transport, or storage. Only 98 mosquito specimens, including 69 *Anopheles*, 23 *Culex,* and six *Aedes* species, were subjected to molecular analysis either to confirm our morphological identification or MALDI-TOF MS. On the other hand, to identify the *Culex* species that were morphologically identified at the genus level and to resolve the discrepancies between the morphological identification and the MALDI-TOF MS identification. Excellent quality MS spectra were obtained for 1,058 out of the 1,253 specimens (84%) including 192/200 for *Aedes*, 589/662 for *Anopheles*, and 277/391 for *Culex*. The blind test showed that 986/997 (99%) of the specimens were correctly identified by MALDI-TOF MS, with LSVs ranging from 1.708 to 2.843. Eleven specimens of *Culex* could not be identified by morphology, MALDI-TOF MS, or molecular analysis. This shows that all techniques

have limitations and that molecular identification requires a reliable genomic sequence and the presence in GenBank of the homologous sequences of the specimen to be identified. Despite the difficulties and limitations of morphological identification, it remains the gold standard when it comes to identifying an unknown specimen, especially when the molecular method cannot identify it due to the absence of its sequence in GenBank or due to the lack of a sequence.

Protocol established for MALDI-TOF MS and molecular analysis of the mosquitoes

Techniques applied in the study
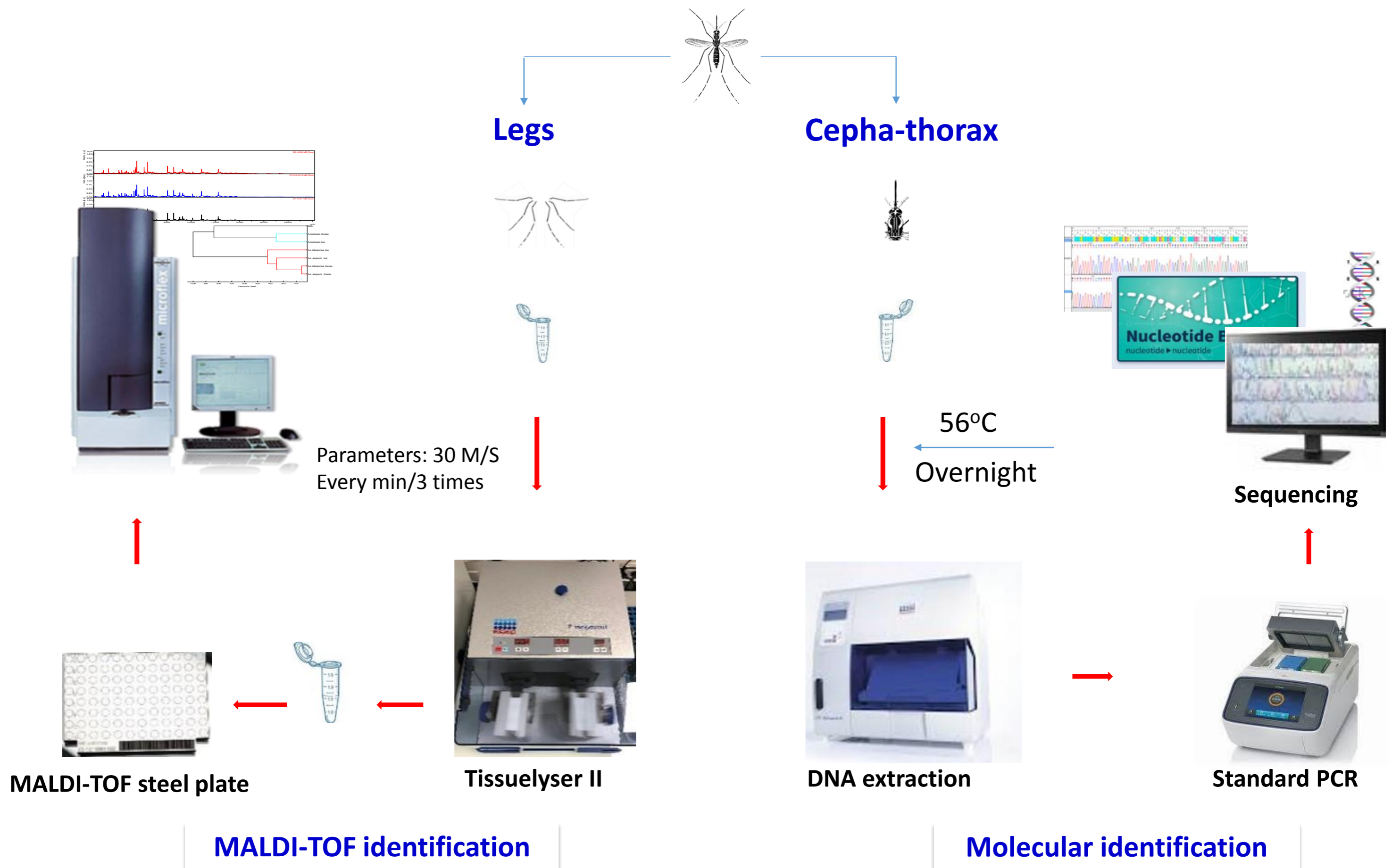
Legs

Cepha-thorax

Parameters: 30 M/S
Every min/3 times

56°C
Overnight

Sequencing

MALDI-TOF steel plate

Tissuelyser II

DNA extraction

Standard PCR

MALDI-TOF identification

Molecular identification

RESEARCH

MALDI-TOF mass spectrometry identification of mosquitoes collected in Vietnam

Ly Na Huynh[1,2,3], Adama Zan Diarra[1,2], Hong Sang Nguyen[3], Long Bien Tran[3], Van Nguyen Do[3], Tran Duc Anh Ly[1,2], Van Hoang Ho[3], Xuan Quang Nguyen[3] and Philippe Parola[1,2*]

Abstract

Background: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a tool that has revolutionised clinical microbiology and has recently been described as an innovative and effective approach to arthropod identification.

Methods: In this study, mosquitoes were captured in Vietnam using four different methods (human landing catch, CDC light traps, BG-Sentinel traps, animal-baited net traps). A total of 4215 mosquitoes were captured and morphologically identified as belonging to three genera: *Aedes*, *Anopheles* and *Culex*. We randomly selected 1253 mosquitoes, including 662 specimens of 14 *Anopheles* species, 200 specimens of two *Aedes* species and 391 morphologically unidentified *Culex* specimens, for molecular and MALDI-TOF MS analysis. The DNA from 98 mosquitoes (69 *Anopheles* specimens, 23 *Culex* specimens and six *Aedes* sp. specimens) was subjected to molecular analysis, either to confirm our morphological identification or the MALDI-TOF MS results, as well as to identify the *Culex* species that were morphologically identified at the genus level and to resolve the discrepancies between the morphological identification and the MALDI-TOF MS identification.

Results: High-quality MS spectra were obtained for 1058 of the 1253 specimens (84%), including 192/200 for *Aedes*, 589/662 for *Anopheles* and 277/391 for *Culex*. The blind test showed that 986/997 (99%) of the specimens were correctly identified by MALDI-TOF MS, with log score values ranging from 1.708 to 2.843. Eleven specimens of *Culex* could not be identified based on morphological features, MALDI-TOF MS or molecular analysis.

Conclusions: This study enabled us to identify several species of mosquitoes from Vietnam using MALDI-TOF MS, and to enrich our database of MALDI-TOF MS reference spectra.

Keywords: MALDI-TOF MS, Molecular identification, Mosquitoes, Vietnam

Background

Mosquitoes are one of the most important insect species in the world and approximately 3500 species are currently recognised [1]. They pose a major threat to human public health and economic development due to their ability to transmit and spread infectious pathogens, such as those causing malaria, dengue fever [dengue virus (DENV)],

yellow fever [yellow fever virus (YFV)], Japanese encephalitis [Japanese encephalitis virus (JEV)], chikungunya [chikungunya virus (CHIKV)] and filariasis [2]. Malaria, caused by *Plasmodium* malarial parasites (i.e. *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*), is transmitted to humans by female *Anopheles* spp. mosquitoes [3–5]. The WHO estimated that there were 229 million cases of malaria in 2019, resulting in 409,000 deaths worldwide [6]. Dengue infection, caused by any of the four DENV serotypes (DENV-1, -2, -3, and -4), is transmitted to humans via the bites of infected female *Aedes aegypti* or *Aedes albopictus* mosquitoes [7]. Dengue is

*Correspondence: philippe.parola@univ-amu.fr
[1] Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France
Full list of author information is available at the end of the article

Huynh *et al. Parasites & Vectors*   (2022) 15:39

Page 2 of 14

epidemic in 128 countries, with four billion people living in areas at risk of dengue infection [8]. There are 100 million cases of dengue annually around the world, resulting in approximately 20,000 deaths [9].

In Vietnam, 255 mosquito species have been described, belonging to 42 subgenera and 21 genera [10]. The majority fall into three genera, namely *Aedes*, *Anopheles* and *Culex*, and include vectors of agents of infectious pathogens [11]. The number of species being discovered is steadily increasing, some of which are difficult to distinguish from others or are morphologically indistinguishable using traditional systematic methods [12]. Molecular biology techniques, such as enzyme electrophoresis and PCR analyses, have been used to discriminate between homogeneous species [13, 14]. However, these techniques are limited due to being time-consuming and expensive and by requiring amplified gene sequences, which are not available in the GenBank databases for certain species [15, 16].

Recent studies show that matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) may be a rapid and accurate tool for the identification of arthropods, their blood meal sources and potentially associated microorganisms [17–19]. Compared to sequencing, MALDI-TOF MS is an easy-to-use, time- and cost-saving technique in entomology, especially if a machine that is generally purchased for microbiology is available [20, 21]. It is a technique based on the identification of proteins contained in a sample of interest. The mass/charge ratio of each protein is measured as it crosses an electromagnetic field after propulsion of the protein molecules by an ultraviolet laser. The mass/charge of the proteins thus measured generates a specific protein mass spectrum of the sample known as the "protein signature". This spectrum is then compared to a reference database containing the spectra of species which have been formally identified morphologically and molecularly [21]. In medical entomology, the choice of the body part of the arthropod and the method of preservation are parameters that can influence the quality of the spectrum [17]. However, the choice of the body part to be used for MALDI-TOF MS analysis depends on the type of arthropod, such as the legs for mosquitoes and ticks [20, 22, 23], the head for bedbugs [24] and the cephalothorax for lice [25]. This proteomic technique has overcome the limitations of traditional morphological and molecular identification methods because it does not require specific entomological knowledge and considerably reduces the time and cost involved [14, 21, 26].

The aim of the present study was to apply MALDI-TOF MS to identify some mosquito species collected in Vietnam in order to first create a reference database for rapid and accurate characterisation.

Methods

Mosquito collection and morphological identification

All mosquitoes were collected by an entomological team from the Institute of Malariology, Parasitology and Entomology in Quy Nhon, Vietnam (IMPE-QN), at the beginning of the rainy season (May) and the beginning of the dry season (January) between 2018 and 2020 in the Central Highlands of Vietnam. The collection area included eight provinces: Hoa Vang (16°03′N; 108°01′E) District, Da Nang Province; Nam Giang (15°65′N; 107°50′E) District, Quang Nam Province; Van Canh (13°37′N; 108°59′E) District, Binh Dinh Province; Dong Xuan (13°22′N; 109°02′E) District, Phu Yen Province; Ea Kar (12°49′N; 108°27′E) District, Dak Lak Province; Krong Pa (13°15′N; 108°45′E) District, Gia Lai Province; Khanh Vinh (12°16′N; 108°53′E) District, Khanh Hoa Province; and Ham Thuan Nam (11°09′N; 108°03′E) District, Binh Thuan Province (Fig. 1).

Collections were performed on eight consecutive nights/days using four different methods: human landing catch (HLC), CDC light trap (CDC-LT), animal-baited net trap and Biogents Sentinel mosquito trap (BG-Sentinel trap; BioGents AG, Regensburg, Germany). Collections took place in the forests, homes and plot huts and around local people's homes in order to capture mosquitoes representing a greater number of genera and species. HLCs were used for night/day collection both indoors and outdoors. The mosquito collectors were local adults who volunteered to capture mosquitoes using HLCs and subsequently trained as mosquito collectors. They were regularly observed for signs of malaria or dengue and were treated if infected. A group of four people worked from 6:00 pm until 12:00 pm sampling *Anopheles* spp. mosquitoes, and from 5:30–7:30 am to 5:30–7:30 pm sampling *Aedes* spp. mosquitoes. Four CDC-LTs and two BG-Sentinel traps were also used for indoor and outdoor overnight captures. They were positioned outdoors with a minimum between-trap distance of 100 m. Indoor traps were placed near bed nets around human sleeping areas. All traps were suspended in the areas targeted from 6:00 pm until 6:00 am. The animal-baited net traps were positioned from 6:00 pm until 6:00 am.

Anopheles and *Aedes* mosquitoes were identified morphologically at the species level and *Culex* mosquitoes were identified at the genus level using Vietnamese mosquito identification keys [27–30]. Only female mosquitoes were used in this study, and captured specimens were stored in silica gel (Carl Roth GmbH, Karlsruhe, Germany) for the most part, or frozen at − 20 °C, before being sent to the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection in Marseille, France for analysis. Samples were stored and subsequently analysed over a period of between 1 and 2.5 years.

Fig. 1 Location of study sites in the Central Highlands of Vietnam
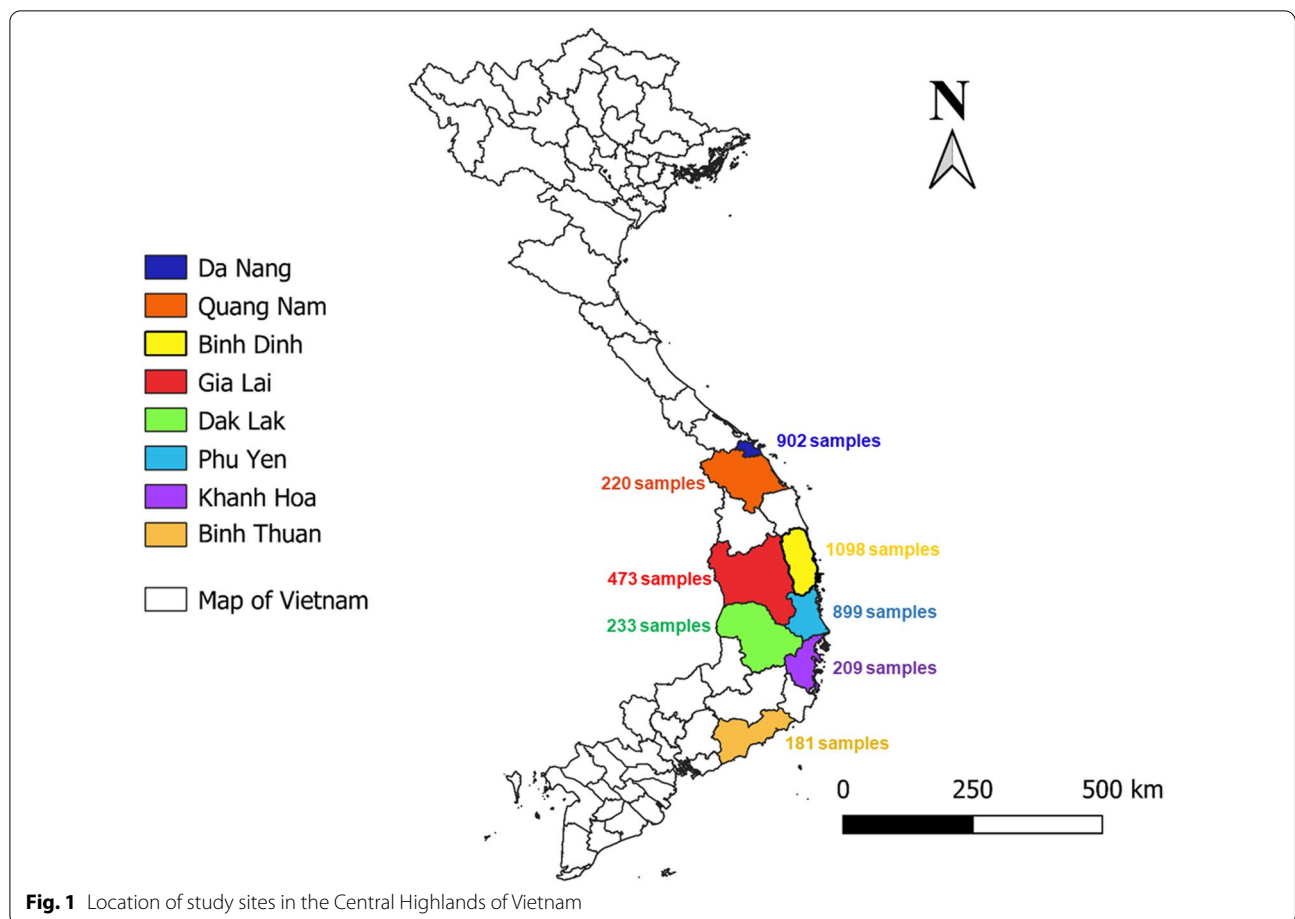
Molecular identification of mosquitoes and phylogenetic analysis

Total DNA was extracted using a BioRobot EZ1 instrument (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Cephalothoraxes from individual mosquitoes were placed in tubes containing 180 µl of G2 buffer and 20 µl of proteinase K and the samples incubated at 56 °C overnight. DNA extraction was performed from 200 µl of the incubation solution, eluted in 100 µl and stored at − 20 °C.

The DNA from 98 mosquitoes, including 43 mosquitoes the spectra of which were used to create our MALDI-TOF MS database and 55 mosquitoes selected for quality control of our MALDI-TOF MS identification, was subjected to molecular identification. The group of 55 mosquitoes selected for quality control of the MALDI-TOF MS identification method consisted of 34 mosquitoes whose identification by MALDI-TOF MS agreed with that by our morphological identification and 21 mosquitoes whose identification by MALDI-TOF MS showed discrepancies with that by our morphological identification. Standard PCR that specifically amplified

a partial 720-bp cytochrome C oxidase I (*COI*) gene [forward primer *COI*_1 (LCO1490): 5′-GGT CAA ATC ATA AGA TAT TGG-3′; reverse primer (HC02198): 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′] was carried out and followed by sequencing [31]. We also sequenced the 720-bp *COI* fragment of 34 specimens of different species of mosquitoes to confirm our MALDI-TOF MS identification using the same primers. Samples for which we did not successfully sequence the *COI*_1 gene were then subjected to another standard PCR and sequencing using the gene known as *COI*_2 [forward primer (CI-J-1632): 5′-TGATCAAAAAATTTATAAT-3′; reverse primer (CI-N-2191): 5′-GGGGTAAAAAAAATA TAAACTTC-3′] amplifying a 560-bp partial sequence [32]. We used the acetylcholinesterase 2 (*Ace2*) gene [direct primer (ACEpip): 5′-GGAAAACAAACGACGT ATGTACT-3′; reverse primer (B1246s): 5′-TGGAGC CTCCTCTTCACGGG-3′] amplifying a 610-bp fragment of *Culex pipiens* and a 274-bp fragment of *Culex quinquefasciatus* to distinguish the two species [33].

For specimens of *Anopheles maculatus* sensu lato (s.l.) that were not identified by MALDI-TOF MS, we

performed standard PCR and sequencing targeting a partial 459-bp internal transcribed spacer 2 (*ITS2*) [forward primer (5.8F): 5′-TGTGAACTG CAGGACACATG-3′; reverse primer (28R): 5′-ATGCTTAAATTTAGGGGG TA-3′] [34].

Standard PCR and sequencing were performed as described in previous studies [23]. Negative controls (single PCR mix and sterile water) and positive controls (DNA extracted from identified *Anopheles gambiae* and *Ae. albopictus* from our laboratory colony) were included in each PCR run. The sequences obtained were assembled and analysed using ChromasPro software (version 1.77) (Technelysium Pty Ltd., Tewantin, Australia) and submitted for analysis to the NCBI BLAST website (http://blast.ncbi.nlm.nih.gov). The phylogenetic tree was built using Mega 7.0 software (https://www.megasoftware.net) [35]. Analyses were performed using the maximum likelihood method proposed by MEGA 7 and bootstrap analyses were performed using 500 replicates.

Preparation of mosquitoes for MALDI-TOF MS analysis

Mosquitoes with at least three legs were selected for the MALDI-TOF MS analysis. Each mosquito was treated individually by grinding the legs in a 1.5-ml Eppendorf tube containing 15 µl of 70% (v/v) formic acid (Sigma-Aldrich, St. Louis, MO, USA) and 15 µl of 50% (v/v) acetonitrile (Fluka, Buchs, Switzerland), with 1.0-mm-diameter glass beads (Sigma France, Lyon, France) using a tissue lyser machine (Qiagen); the optimal parameters had been previously established [36]. The crushed legs were centrifuged, and a 1-µl aliquot of the supernatant of each sample was deposited in quadruplicate on a MALDI-TOF MS steel plate (Bruker Daltonics, Wissembourg, France) and covered after drying with a matrix solution composed of 1 µl of saturated alpha-cyano-4-hydroxycinnamic acid (Sigma France), 50% acetonitrile (v/v), 2.5% trifluoroacetic acid (v/v) (Sigma-Aldrich Co. Ltd., Gillingham Dorset, UK) and high-performance liquid chromatography-grade water [19, 37]. The target plate was then air-dried for a few minutes at room temperature before being introduced into the Microflex LT MALDI-TOF MS apparatus (Bruker Daltonics) for analysis. The quality of the matrix, the sample loading and the performance of the MALDI-TOF MS apparatus were controlled using the leg of an *Ae. albopictus* from our laboratory as a positive control.

MALDI-TOF MS parameters

The spectral profiles obtained from the mosquito legs were visualised using a Microflex LT MALDI-TOF mass spectrometer with FlexControl software (version 3.3; Bruker Daltonics). The spectra were acquired in a positive linear mode at a laser frequency of 50 Hz. The accelerating voltage was 20 kV, and the extraction delay time was 200 ns. Each spectrum corresponded to the ions obtained from the 240 laser shots performed in six regions at the same location and acquired automatically using the AutoXecute Flex Control software (version 2.4; Bruker Daltonics). The MS profiles were visualised using the FlexAnalysis v3.4 software, MALDI Biotyper Compass Explorer v4.1.70 (Bruker Daltonics), and ClinPro-Tools v3.0 software (Bruker Daltonics) was used for data processing.

Spectral analysis and reference database creation

All spectra were visualised using the FlexAnalysis v.3.3 software to check their quality, reproducibility, and specificity. Spectra of poor quality, i.e. those which had low intensity (< 3000 AU), which were non-reproducible and which featured background noise, were excluded from the study. Finally, cluster analysis was performed to visualise the reproducibility and specificity of the spectra using the Main Spectrum Profile (MSP) dendrogram function of the MALDI Biotyper Compass Explorer v4.1.70 software. The dendrogram was performed using two to three spectra per species, excluding *Anopheles barbirostris* and *Culex pallidothorax*, for which there was only one spectrum for each species. The MS dendrogram is the result of comparing the MSPs produced by the MALDI Biotyper software and clustered according to the mass profile of the proteins (i.e. their mass signals and intensities), and this MS dendrogram illustrates how the samples are related to one another. The MS dendrograms helped us select representative reference spectra of the species [23]. Between one and four reference spectra per species were then added to our in-house arthropod database of MS reference spectra [20]. However, the reference spectra of *Anopheles kochi* and *Anopheles jeyporiensis* were not added to the database as the identification of *An. kochi* had not been molecularly confirmed and the spectrum of *An. jeyporiensis* was of poor quality.

Blind test for mosquito identification

The MS spectra from *Ae. albopictus*, *Ae. aegypti* and *Culex* spp. were blind tested against our pre-existing database containing spectra from *Ae. albopictus*, *Ae. aegypti*, *Cx. quinquefasciatus* and *Culex sitiens* species, the reference spectra of which were already available in our MALDI-TOF MS database. For specimens of *Culex* spp. that were not identified by this first blind test and the *Anopheles* species, a blind test was performed with the remaining spectra after creating a database of reference spectra of the different species confirmed by molecular biology. A total of 43 specimens, including between one and four of each species, were used to create the MALDI-TOF MS spectral database of Vietnamese mosquitoes.

The level of reliability of species' identification was determined using log score values (LSVs) of between 0 and 3 using the MALDI-TOF Biotyper software, which corresponded to a degree of similarity between the queried spectra and the reference spectrum in the database. The LSV threshold score set for correct identification of mosquito species was 1.70 in this study as this is the optimal threshold value applied for arthropod identification using MALDI-TOF MS [38, 39].

Results

Mosquito collection and morphological identification

A total of 4215 individual mosquitoes were captured using different methods, including 1301 specimens (30.9%) by HLCs, 1335 (31.7%) by CDC-LTs, 891 (21.1%) by animal-baited net traps and 688 (16.3%) by BG-Sentinel traps. Mosquitoes collected by CDC-LTs, HLCs and animal-baited net traps were more abundant in quantity and number of species than those caught using the BG-Sentinel traps. The number of specimens collected per province was: Da Nang ($n=902$), Quang Nam ($n=220$), Binh Dinh ($n=1098$), Gia Lai ($n=473$), Dak Lak ($n=233$), Phu Yen ($n=899$), Khanh Hoa ($n=209$) and Binh Thuan ($n=181$) (Table 1; Fig. 1; Additional file 1: Table S1). Two *Aedes* species were identified

morphologically: *Ae. aegypti* ($n=507$; 12%) and *Ae. albopictus* ($n=640$; 15.2%). Fourteen *Anopheles* species were identified based on morphological characteristics: *Anopheles aconitus* ($n=15$; 0.4%), *Anopheles annularis* ($n=3$; 0.1%), *Anopheles barbirostris* ($n=7$; 0.2%), *Anopheles dirus* s.l. ($n=281$; 6.7%), *An. kochi* ($n=8$; 0.2%), *An. maculatus* s.l. ($n=1062$; 25.2%), *Anopheles minimus* s.l. ($n=183$; 4.2%), *An. jeyporiensis* ($n=1$; 0.02%), *Anopheles peditaeniatus* ($n=81$; 1.9%), *Anopheles sinensis* ($n=11$; 0.2%), *Anopheles splendidus* ($n=36$; 0.8%), *Anopheles jamesii* ($n=49$; 1.2%), *Anopheles vagus* ($n=35$; 0.8%) and *Anopheles varuna* ($n=9$; 0.2%). The *Culex* mosquitoes, representing 1287 mosquitoes (30.7%), were not identified to the species level when specific morphological characters could not be identified with certitude due to being damaged during collection, transport or storage.

MALDI-TOF MS identification of the mosquitoes

Of the 4215 mosquitoes collected, 1253 (30%) mosquitoes, including 662 specimens of *Anopheles* spp., 391 specimens of *Culex* spp. and 200 specimens of *Aedes* spp., were randomly selected for MALDI-TOF MS analysis (Table 2). Overall, good-quality MS spectra were obtained from the legs of 1058 of the 1253 (84%) mosquitoes subjected to MALDI-TOF MS analysis. The

Table 1 The number and composition of the mosquitoes collected by different methods in Vietnam between May 2018 and January 2020

Morphological ID	Different collection methods									
	Human-landing catch		Animal-baited net trap		CDC-LT		BG-Sentinel trap		Total	
	No. collected	%	No. collected	%	No. collected	%	No. collected	%	Total number collected	Total %
Aedes albopictus	403	9.6	0	0	0	0	237	5.6	640	15.2
Ae. aegypti	261	6.2	0	0	0	0	246	5.8	507	12
Anopheles aconitus	3	0.1	5	0.1	7	0.2	0	0	15	0.4
An. annularis	0	0	2	0.1	1	0.02	0	0	3	0.1
An.barbirostris	0	0	3	0.1	4	0.1	0	0	7	0.2
An. dirus	187	4.4	23	0.6	71	1.7	0	0	281	6.7
An. kochi	0	0	3	0.1	5	0.1	0	0	8	0.2
An. jamesii	12	0.3	12	0.3	25	0.6	0	0	49	1.2
An. jeyporiensis	0	0	1	0.02	0	0	0	0	1	0.02
An. maculatus	323	7.7	289	6.9	450	10.7	0	0	1062	25.2
An. minimus	83	1.9	34	0.8	66	1.5	0	0	183	4.2
An. peditaeniatus	0	0	45	1.1	36	0.8	0	0	81	1.9
An. sinensis	0	0	6	0.1	5	0.1	0	0	11	0.2
An. splendidus	8	0.2	22	0.5	6	0.1	0	0	36	0.8
An. vagus	7	0.2	26	0.6	2	0.1	0	0	35	0.8
An. varuna	3	0.1	4	0.1	2	0.04	0	0	9	0.2
Culex spp.	11	0.3	416	10	655	15.5	205	4.9	1287	30.7
Total	4215									

Huynh *et al. Parasites & Vectors*   (2022) 15:39

Page 6 of 14

Table 2 Number of specimens of each morphologically identified mosquito species randomly selected for MALDI-TOF MS analysis, creation of the reference database and blind test results for MS identification and log-score values of each species

Morphological ID	No. tested/no. collected	No. of good spectra	No. added to database	MALDI-TOF MS ID	LSV range (minimum–maximum)
Ae. albopictus	100/640	94	0	*Ae. albopictus*[a] (94)	1.892–2.371
Ae. aegypti	100/507	98	0	*Ae. aegypti*[a] (98)	1.729–2.39
An. aconitus	10/15	7	2	*An. aconitus* (5)	1.732–2.786
An. annularis	2/3	2	1	*An. annularis* (1)	2.082
An. barbirostris	3/7	1	1	*An. barbirostris* (0)	Not applicable
An.dirus	79/281	79	3	*An. dirus* (75)	1.78–2.782
An. kochi	7/8	0	0	Not identified	Not applicable
An. jamesii	36/49	34	4	*An. jamessi* (29)	1.844–2.605
An. jeyporiensis	1/1	0	Not applicable	Not applicable	Not applicable
An. maculatus	329/1062	287	2	*An. maculatus* (276)	1.708–2.782
An. minimus s.l.	79/183	7	1	*An. minimus* (4)	2.174–2.815
		71	3	*An. harrisoni* (66)	1.836–2.821
An. peditaeniatus	46/81	43	4	*An. peditaeniatus* (37)	1.814–2.539
An. sinensis	10/11	8	1	*An. sinensis* (7)	1992–2.755
An. splendidus	36/36	28	2	*An. splendidus* (24)	1.851–2.449
An. vagus	16/35	14	3	*An. vagus* (11)	2.088–2.777
An. varuna	8/9	8	1	*An. varuna* (7)	2.065–2.449
Culex sp.	391/1287	277	0	*Cx. quinquefasciatus*[a] (48)	1.725–2.401
Culex sp.			4	*Cx. tritaeniorhynchus* (58)	1.739–2.338
Culex sp.			4	*Cx. pseudovishnui* (46)	1.820 –2.012
Culex sp.			1	*Cx. pallidothorax* (0)	0
Culex sp.			2	*Cx. fuscocephala* (25)	1.756–2.843
Culex sp.			4	*Cx. vishnui* (44)	1.72–2
Culex sp.			0	*Cx. sitiens*[a] (30)	1.737–2.144
Culex spp.			Not applicable	Not identified	Not applicable
Total	1253/4215	1058	43		

[a] Species was already available in our database

( ) the values in parentheses: the number of specimens of each species was identified using MALDI-TOF MS

percentage of good-quality spectra was highest for *Aedes* spp., with 96% (192/200) good-quality spectra, followed by *Anopheles* spp. (88.97%; 589/662) and *Culex* spp. (70.84%; 277/391). Visually, the comparison of MS profiles of legs of different species using FlexControl analysis revealed intra-species reproducibility and inter-species specificity (Additional file 2: Fig. S1). The MS protein profiles of between two and three specimens of each mosquito species used to generate an MSP dendrogram showed a clustering of specimens of the same species on the same branch (Fig. 2). The average discriminant peak intensity of 19 mosquito species, excluding those with low numbers, is presented in Additional file 3: Table S2.

Querying 192 good-quality spectra of *Aedes* spp. against our in-house MALDI-TOF MS database showed that 94 spectra were *Ae. albopictus* and 98 were *Ae. aegypti*, with LSVs ranging from 1.729 to 2.39 (Table 2). For *Anopheles* spp., we added 28 reference spectra from

between one and four spectra of each species, confirmed by molecular biology analysis, to our MALDI-TOF MS database, which did not contain spectra of the concerned species. The remaining 561 spectra were then blind tested against the updated database, revealing that 96.8% (543/561) had been correctly identified, i.e. presented a match between our morphological identification and MALDI-TOF MS results. The LSVs of these *Anopheles* spp. specimens ranged from 1.708 to 2.821 (Table 2). For the remaining 18 specimens (3.2%) there were discrepancies between our morphological identification and the MALDI-TOF MS identification. Molecular biology analysis confirmed the MALDI-TOF MS identification of all species, with the exception of specimens of *An. maculatus* s.l. The dendrogram visually produced using the spectra of mosquitoes identified as *An. maculatus* s.l. shows that they form two distinct branches (Fig. 3).

Huynh *et al. Parasites & Vectors*   (2022) 15:39

Page 7 of 14

Fig. 2 Dendrogram constructed from between two and three representative leg spectra of different mosquito species collected in Vietnam between May 2018 and January 2020. The dendrogram was created using Biotyper v3.0 software and the distance units correspond to the relative similarity of the MS spectra. The red lines show the cut-off level between mosquito genera. The purple lines show the cut-off level between the different *Anopheles* species. The blue and green lines show the cut-off between *Culex* and *Aedes* species, respectively
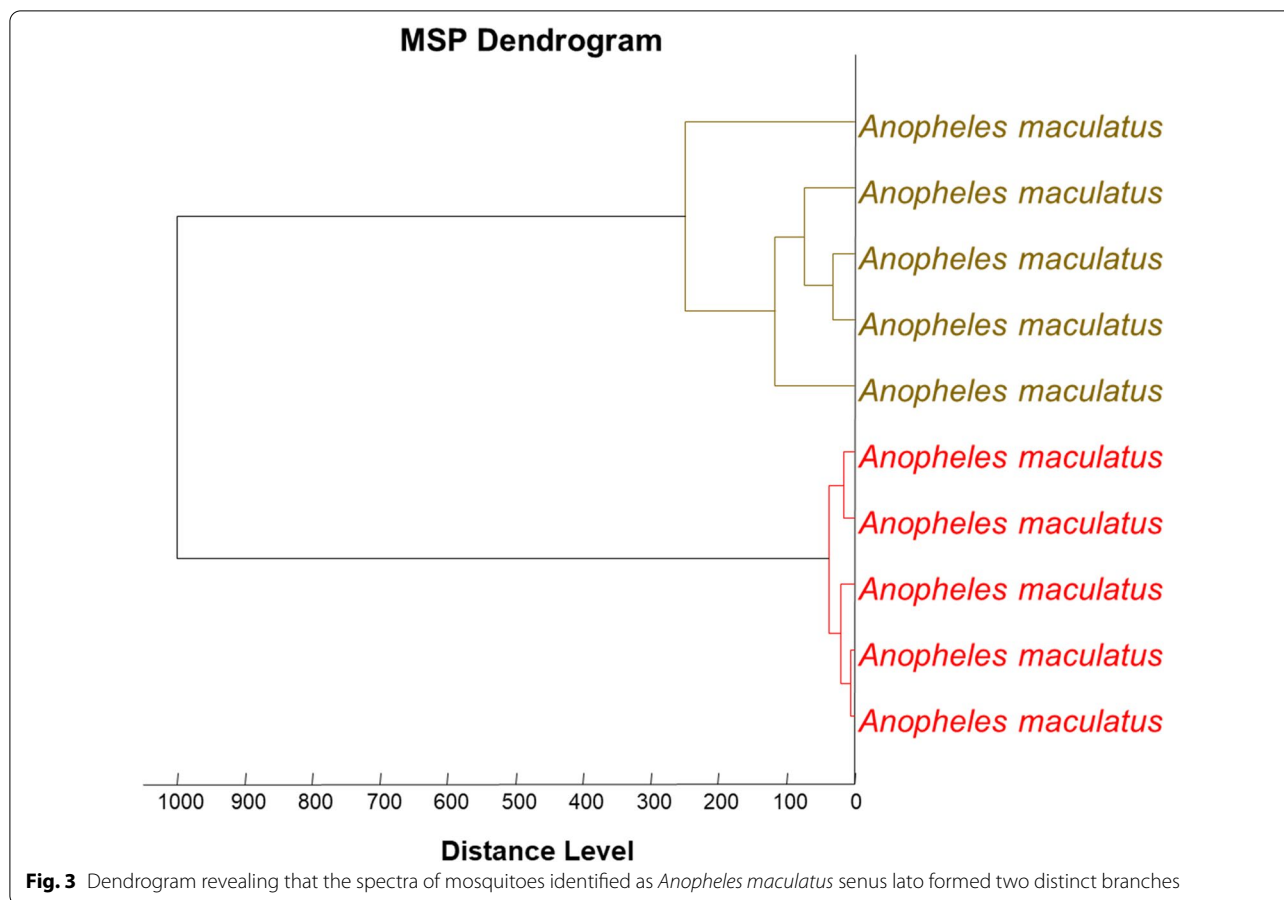
Querying the 277 legs of *Culex* spp. against our database, which contained the spectra of 17 *Culex* species, showed that 28.2% (78/277) were identified, including 48 *Cx. quinquefasciatus* and 30 *Cx. sitiens*, with LSVs ranging from 1.725 to 2.401, and from 1.737 to 2.144, respectively. From the remaining 199/277 legs of *Culex* spp. which were unidentified, we randomly selected 15 of the 199 *Culex* specimens to create our database with reference spectra of four *Culex tritaeniorhynchus*, four *Culex pseudovishnui*, four *Culex vishnui* s.l., two *Culex fuscocephala*, and one *Culex pallidothorax* identified by molecular biology. The second blind test of the remaining 184 spectra against the updated database showed that 92.4% (170/184) were identified, with LSVs ranging from 1.739 to 2.843. Of the 173 *Culex* mosquitoes which were identified, 58 specimens were *Cx. tritaeniorhynchus*, 46 were *Cx. pseudovishnui*, 44 were *Cx. vishnui* s.l. and 25 were *Cx. fuscocephala* (including 3 specimens which showed discrepancies between the morphological and the MALDI-TOF MS results, which were subsequently confirmed by molecular analysis). Finally, 11 specimens with good spectra were not identified by MALDI-TOF MS and could not be identified molecularly because we could not sequence them using the three types of genes (Additional file 4: Fig. S2).

Molecular identification of the mosquitoes

Sequencing and BLAST of the *COI*_1 or *COI*_2 gene sequences from the *Anopheles* and *Aedes* species selected for the database revealed reliable and consistent species' identification according to the morphological identification, with between 98.4 and 100% identity with GenBank sequences (Table 3). The *COI*_1 gene was used to identify species of 15 *Culex* spp. randomly selected to enter in our database. BLAST showed that four were between 99.3% and 100% identical to *Cx. tritaeniorhynchus* (MF179219; MF179221; MF179231; MF179232), four were between 97.9% and 98.2% identical to *Cx. pseudovishnui* (AB738092; LC054483), four were between 99.2% and 100% identical to *Cx. vishnui* (MF179240), two were 100% identical to *Cx. fuscocephala* (HQ398887) and one was 98% identical to *Cx. pallidothorax* (MF179281). Only one *Cx. quinquefasciatus* species could not be identified using *COI*_1 DNA barcoding; this species was then identified using the *Ace*2 gene, which was shown to share between 99.6% and 100% identity with GenBank (MN299021; MK575480) (Table 3).

Similarly, for specimens that showed discrepancies between our morphological identification and MALDI-TOF MS identification, i.e., *An. aconitus* identified as *An. varuna* by MALDI-TOF MS, *An. dirus* identified as *An. sinensis*, *An. minimus* identified as *An. varuna*, *An. jamesi* identified as *An. maculatus*, *An. pediteniatus* identified as

19

Huynh *et al. Parasites & Vectors*   (2022) 15:39

Page 8 of 14

Fig. 3 Dendrogram revealing that the spectra of mosquitoes identified as *Anopheles maculatus* senus lato formed two distinct branches

An. sinensis, and *Culex* sp. identified as *An. minimus*, *An. jamesii*, and *An. dirus*. We found consistent identification between MALDI-TOF MS and molecular tools after querying the sequences against the GenBank database. However, for some specimens that were morphologically identified as *An. maculatus* s.l., the *COI*_1 sequences were 93.44% identical to *An. maculatus* (KT382822). We had no sequence for these specimens with the *ITS* gene generally used to differentiate between species of the *An. maculatus* complex (Table 3).

Finally, interrogation of the sequences from the samples selected for confirmation showed a perfect match between MALDI-TOF MS and molecular identification (Tables 2 and 3).

The *COI*_1, *COI*_2 or *Ace2* sequences of some specimens were deposited in GenBank, and the names and accession numbers are listed in Additional file 5: Table S3. The phylogenetic tree constructed using the *COI*_1 sequences and their homologues available on GenBank shows that these 22 mosquito species formed distinct groups (Fig. 4) while correlated with morphological and MALDI-TOF MS identifications.

Discussion

MALDI-TOF MS is an innovative tool for the rapid and accurate identification of arthropods [21]. Here, we report the first MALDI-TOF MS reference spectra of a selection of protein extractions from Vietnamese mosquito legs. These species are widely distributed, medically important mosquitoes, and were most frequently captured during entomological surveillance and management programmes in the Central Highlands of Vietnam. Among them, *An. minimus* s.l., *An. dirus* s.l. and *An. maculatus* s.l. are considered to be the main vectors of human malaria in Vietnam [40–42] and other parts of Southeast Asia, such as Laos, Cambodia and Thailand [28, 43, 44]. *Aedes albopictus* and *Ae. aegypti*, are vectors of DENV, and recent epidemics of dengue fever in 2017 and 2019 resulted in 183,287 and 200,000 cases, with 32 and 50 deaths, respectively [45–47]. These two *Aedes* species are also important vectors of the CHIKV and Zika virus, both of which are present in the arbovirus burden of disease in Vietnam [48]. Mosquitoes of the genus *Culex* are the main vectors responsible for the transmission of JEV throughout Vietnam [49]. Despite the introduction of a JEV vaccine in 1997 and mandatory

Table 3 Details of the result of the specimens submitted to molecular biology analysis and homology with the reference sequences available on GenBank using BLAST

Mosquito species (morphological ID)	No. sequenced	Percentage of similarity between sequences of same species	Molecular identification (% identity)	Accession no.
-Specimens using for confirming MALDI-TOF MS identification				
Ae. aegypti[a]	3	100%	*Ae. aegypti* (100%)	MN299016
Ae. albopictus[a]	3	99.7–100%	*Ae. albopictus* (99.7–100%)	MN299017, MK995332
Cx. quinquefasciatus[b]	3	99–100%	*Cx. Quinquefasciatus* (99.6–100%)	MN299021, MK575480
Cx. sitiens[a]	2	100%	*Cx. sitiens* 99.8%	MF179212
An. aconitus[a]	2	99.8%	*An. aconitus* (100%)	HQ877378
An. annularis[a]	1	99.5%	*An. annularis* (96.6%)	KF406655
An. dirus[a]	2	100%	*An. dirus* (99.7%)	JX219732
An. minimus s.l[a]	2	100%	*An. harrisoni* (99.8–100%)	HQ877377
An. minimus s.l.[a]	2	100%	*An. minimus* (99.4%)	HQ877337
An. jamessi[a]	2	89.2–100%	*An. jamessi* (99.7–99.8%)	MT380518, MT871938
An. pediteaniatus[a]	3	96–99.5%	*An. pediteaniatus* (99.3–99.8%)	LC333267
An. sinensis[a]	2	100%	*An. sinensis* (99.7%)	MG816536
An. vagus[a]	2	100%	*An. vagus* 99.7%	MK685245
An. maculatus[a]	3	100%	*An. maculatus* 99.8%	KT382822
An. varuna[a]	2	100%	*An. varuna* (100%)	HQ877380
Specimens used for database update				
An. aconitus[a]	2	90.4–99.8%	*An. aconitus* (99.8–100%)	HQ877378
An. annularis[a]	1	-	*An. annularis* (96.6%)	KF406655
An. dirus[a]	3	89.7–100%	*An. dirus* (99.5–99.7%)	JX219732
An. minimus s.l.[a]	4	90–99.84%	*An. harrisoni* (99.8–100%)	HQ877377
			An. minimus (99.4%)	HQ877337
An. barbirostris[a]	1	99.7%	*An. barbirostris* (99.7%)	AB971312
An. jamessi[a]	4	89.2–100%	*An. jamessi* (99.7–99.8%)	MT380518, MT871938
An. pediteaniatus[a]	4	96–99.5%	*An. pediteaniatus* (99.3–99.8%)	LC333267
An. sinensis[a]	1	98.7–98.9%	*An. sinensis* (99.5–99.7%)	MG816536, MG816562, KX779641
An. vagus[a]	3	98.2–98.7%	*An. vagus* (99–99.7%)	MK685245, MH425442, MF179262
An. maculatus[a]	2	100%	*An. maculatus* 99.8%	KT382822
An. varuna[a]	1	-	*An. varuna* (100%)	HQ877380
An. splendidus[c]	2	99.8%	*An. splendidus* (98.4–98.7%)	MK685253
Culex spp.[a]	15		*Cx. tritaeniorhynchus* (99.3–100%)	MF179219, MF179221, MF179231, MF179232
			Cx. fuscocephala (100%)	HQ398887
			Cx. pseudovishnui (97.9–98.2%)	AB738092, LC054483
			Cx. pallidothorax (98%)	MF179281
			Cx. vishnui (99.2%)	MF179240
Specimens with discrepancies between morphological and MALDI-TO MS identification				
An. aconitus[a]	1	-	*An. varuna* (100%)	HQ877380
An. dirus[a]	1	-	*An. sinensis* (99.5%)	MG816562
An. minimus[a]	4	99–100%	*An. varuna* (99–100%)	HQ877380
An. jamesi[a]	1	-	*An. maculatus* (93.8%)	KT382822
An. pediteniatus[a]	2	99.5%	*An. sinensis* (99.4–99.5%)	MG816562, KX779641
An. maculatus[a]	9	100%	*An. maculatus* (93.6%)	KT382822
Culex sp.[a]	1	-	*An. minimus* A (99.5%)	HQ877337
Culex sp.[a]	1	-	*An. jamesii* (99.7%)	MT871938
Culex sp.[a]	1	-	*An. dirus* (99.7%)	JX219732

[a] *COI*_1 gene

[b] *Ace2* gene

[c] *COI*_2 gene

Huynh *et al. Parasites & Vectors*   (2022) 15:39

Page 10 of 14

vaccination of children under the age of 5 years in Vietnam [49] over the past 10 years, the average annual number of JEV cases is between 1000 and 1200, with 20 to 50 deaths [50].

In terms of entomological surveillance, all mosquito species collected using the four methods in this study did not really have much of an impact on the quality of the MALDI-TOF MS profile, despite the duration of exposure to the mosquitoes according to temperature and humidity varying widely. After 12 h of use of the animal-baited net trap, the CDC-LT and the BG-sentinel trap, compared to the HLC method, the LSV median values remained at the optimal threshold score (i.e. 1.7). The stability of the MALDI-TOF MS results could likely be explained by the mosquitoes being handled carefully before being stored in dry tubes with silica gel.

Two species of *Aedes* and 14 species of *Anopheles* were identified based on morphological characteristics. In contrast, *Culex* specimens were only identified to the genus level based on morphological features due to the integrity of the mosquitoes, which did not allow for discrimination between morphologically identical sibling mosquito species and cryptic species groups. Although morphological identification is the "gold standard" for distinguishing mosquitoes based on external characteristics, it is unable to separate species belonging to the same complex. In the present study, almost all of the *Culex* species that were identified belong to species complexes that are not only difficult to distinguish morphologically [51] but also molecularly [23, 39, 52].

High-quality MS spectra were obtained for 84% of mosquito samples analysed by MALDI-TOF MS. The quality of the spectra obtained from *Aedes* spp. was higher than that obtained for MS spectra from *Anopheles* spp. and *Culex* spp. However, our percentage of good-quality spectra was lower than that found in a study of mosquitoes stored at − 20 °C [53]. This is due to the difference in storage methods, as demonstrated by previous studies which have highlighted that storage at − 20 °C is the best method of preserving arthropods for MALDI-TOF MS analysis for periods not exceeding 6 months [54] while for MALDI-TOF MS analyses that are to be performed after periods exceeding 6 months, the best method of storage is − 80 °C [39]. However, many laboratories do not have a − 80 °C freezer for mosquito storage, and in these cases − 20 °C freezing is the optimal method. In our study, conducted under field conditions, the percentage of good-quality

spectra was higher than that of another study on mosquitoes which were also preserved in silica gel [20].

The MS spectra generated from the legs of the different mosquito species and the dendrogram show intra-species reproducibility and inter-species specificity or grouping of specimens of the same species on the same branch (Fig. 2; Additional file 2: Figure S1). This specificity of the MALDI-TOF MS spectra of mosquitoes has already been reported [20, 23, 53].

MALDI-TOF MS analysis allowed us to identify 22 species of mosquitoes, with LSV scores ranging from 1.708 to 2.843, including 18 species that were not in our MALDI-TOF MS database. Two species of *Anopheles* (*An. kochi* and *An. jeyporiensis*) were identified based on morphological features and 11 specimens of *Culex* sp. were not identified by MALDI-TOF MS because their spectra were not in our database. The latter is due to *An. kochi* and *Culex* not being molecularly identified in our study because we did not obtain DNA sequences from them, despite having good-quality spectra. An arthropod spectrum is added to our database only when the specimen is unambiguously identified by morphology and molecular biology analysis [17]. We had only one specimen of *An. jeyporiensis* for which the MS spectrum was of poor quality [lower intensity: < 3000 AU; which was non-reproducible and which contained background noise). In addition to being able to identify closely related mosquito species, we found that MALDI-TOF MS can distinguish between subgroups of species of the same complex, which are impossible to distinguish morphologically, as was the case for the *An. minimus* complex (*An. minimus* and *An. harrisoni* formerly referred to as *An. minimus* A and C) [55, 56] and for the *An. maculatus* s.l. complex (Figs. 2, 3). Interestingly, the first two top hits for species classification were *Cx. quinquefasciatus* and *Cx. pipiens*, both ranging from 99 to 100% of sequence correspondence. These *Cx. quinquefasciatus* specimens were then classified by *Ace*_2 (Table 3). Preliminary studies had already reported that the MALDI-TOF MS technique is capable of identifying arthropods at the species, species subgroup or complex level [20, 57].

The morphological identification of all the specimens introduced in our database was confirmed by molecular biology using at least one gene. We found consistency between our morphological and molecular identifications, as our sequences had identities ranging from 96.6 to 100% with their GenBank homologues. For specimens

(See figure on next page.)
Fig. 4 Neighbour-joining tree of genetic relationships obtained from *COI*_1 DNA barcode from 20 mosquito species collected in Vietnam (not including *Anopheles splendidus* by *COI*_2 and *Culex quinquefasciatus* by *Ace*2 gene) compared to other reference sequences (highlighted in dark text) in the GenBank database based on the 720-bp partial *COI*_1 gene. For the sequences downloaded from the GenBank database, the species names are indicated with their GenBank accession numbers and the country of origin, followed by the year they were deposited in GenBank. Node numbers are percentages of bootstrap values
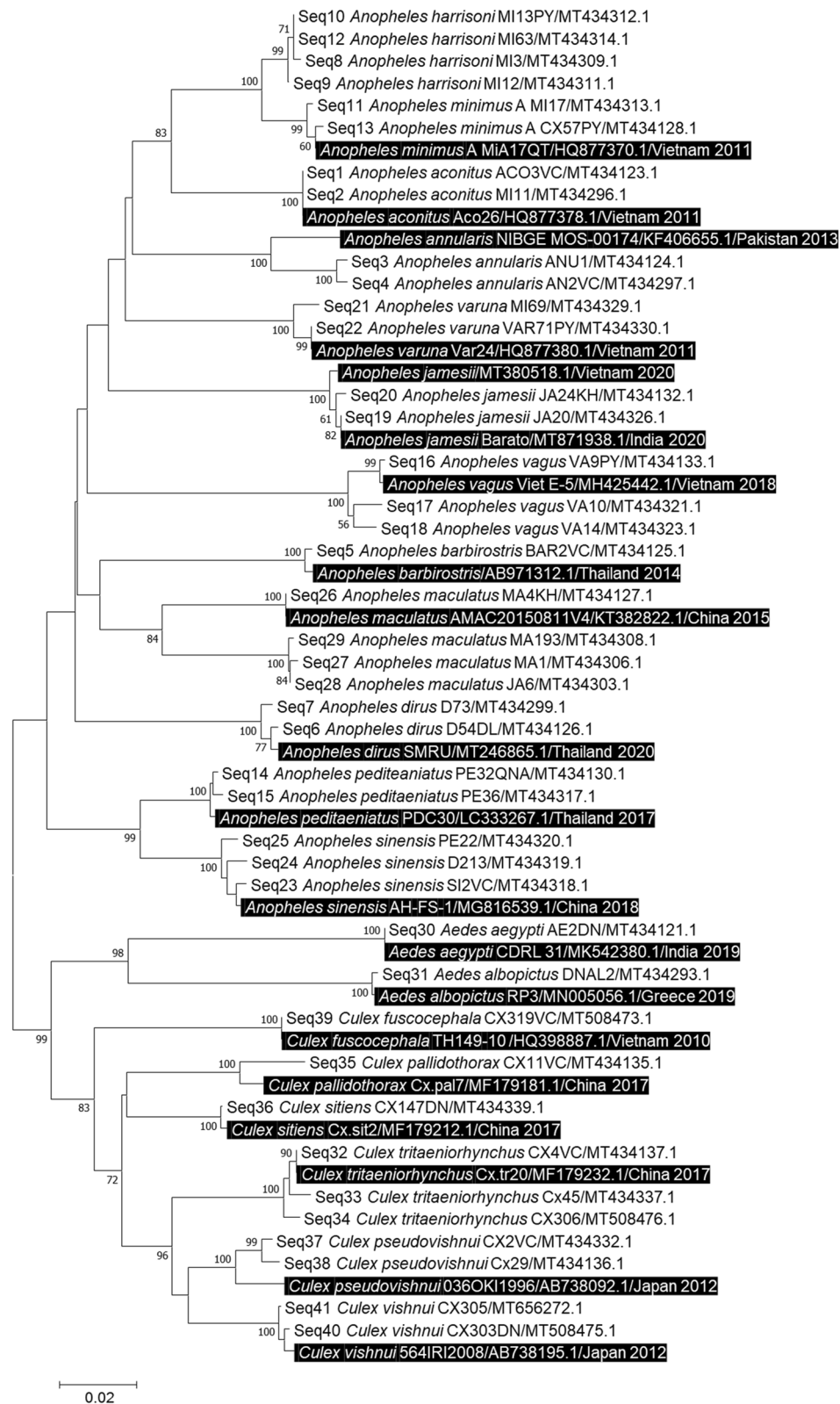
Huynh *et al. Parasites & Vectors*   (2022) 15:39

Page 11 of 14

Fig. 4 (See legend on previous page.)

Huynh *et al. Parasites & Vectors*   (2022) 15:39

Page 12 of 14

that showed inconsistencies between morphological identification and MALDI-TOF MS identification, molecular biology analysis agreed with the MALDI-TOF MS identification. Morphological identification errors thus occurred, showing the limitations of this method. These errors were partly identified by MALDI-TOF MS, which we consider to be reliable for the identification of arthropods. Sequencing, which is the last technique we used to either confirm morphological and MALDI-TOF MS identification or to separate the two, did not allow us to identify 11 *Culex* specimens for which we had no morphological and MALDI-TOF MS identification of the species. This shows that all techniques have limitations and that molecular identification requires a reliable genomic sequence and the presence in GenBank of the homologous sequences of the specimen to be identified [26]. Despite the difficulties and limitations of morphological identification, it remains the gold standard when it comes to identifying an unknown specimen, especially when the molecular method cannot identify it due to the absence of its sequence in GenBank or due to the lack of a sequence.

Conclusion

This study is the first to apply MALDI-TOF MS to the identification of field-caught mosquitoes in Vietnam, where the burden of disease transmitted by these vectors is high. It confirms that misidentification of mosquitoes by morphology can be prevented with MALDI-TOF MS, provided that the reference spectrum of that mosquito species is present in the MALDI-TOF MS database. The study allowed us to enrich our in-house MALDI-TOF MS database by adding reference spectra of new species. MALDI-TOF MS is a promising entomological surveillance tool that can significantly contribute to the control of mosquito-borne diseases in Vietnam.

Abbreviations

BG-Sentinel: Biogents Sentinel mosquito trap; BLAST: Basic Local Alignment Search Tool; CDC-LT: US Centers for Disease Control and Prevention miniature light traps; ID: Identification; LSVs: Log score values; MALDI-TOF MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-022-05149-2.

Additional file 1: Table S1. Distribution of the adult mosquito species collected in different regions of Vietnam.

Additional file 2: Figure S1. Comparison of MALDI-TOF MS spectra from the legs of 22 mosquito species collected in Vietnam between May 2018 and January 2020. MS spectra revealed intra-species reproducibility and inter-species specificity. Abbreviations: a.u., Arbitrary units; m/z, mass to charge ratio

Additional file 3: Table S2. List of the top ten average mass peaks of the different mosquito species. The top ten average mass peaks by mosquito species are shown in bold. The average intensity of the mass peaks is indicated using a colour scale from green to red, indicating moderate and high average peak intensity, respectively. The list of mass peaks used to distinguish different mosquito species is based on the analysis of the ClinProTools genetic algorithm model. Abbreviations: Da, Daltons

Additional file 4: Figure S2. Flow diagram of mosquito specimens which were included, analysed, and added to our in-house database using MALDI-TOF MS and molecular tools

Additional file 5: Table S3. Accession numbers of sequences of different species of mosquitoes deposited in GenBank

Authors' contributions

LNH: collection and morphological identification of mosquito specimens, methodology, data analysis and writing of original draft. AZD: methodology, data analysis, and writing of original draft. HSN, LBT and VND: collection and morphological identification of mosquito specimens. TDAL: methodology and data analysis. VHH and XQN: reviewing the manuscript. PP: methodology, visualisation, supervision and writing and reviewing of the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

All relevant data are presented in the paper and its supporting ESM files. Our MALDI-TOF MS database is publicly available on our laboratory website and can be downloaded with the following DOI number: https://doi.org/10.35081/hwtr-5224.

Declarations

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of the Institute of Malariology, Parasitology and Entomology in Quy Nhon, on behalf of the Vietnam Ministry of Health (Approval Number 308/VSR-CT-2018). For ethical approval regarding human landing catches (HLC), we obtained consent from local adult volunteers. With regard to animals that were used for animal-baited net traps, such as domestic cows, we obtained permission from the owners of the cows.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1] Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France. [2] IHU-Méditerranée Infection, Marseille, France. [3] Institute of Malariology, Parasitology and Entomology Quy Nhon (IMPE-QN), Quy Nhon, Vietnam.

Huynh *et al. Parasites & Vectors*   (2022) 15:39

Page 13 of 14

References

1. Xia H, Wang Y, Atoni E, Zhang B, Yuan Z. Mosquito-associated viruses in China. Virol Sin. 2018;33:5–20.
2. World Health Organization (WHO). Vector-borne diseases. 2017. https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases. Accessed 06 Jan 2021.
3. Nakazawa S, Marchand RP, Quang NT, Culleton R, Manh ND, Maeno Y. *Anopheles dirus* co-infection with human and monkey malaria parasites in Vietnam. Int J Parasitol. 2009;39:1533–7.
4. Van Long B, Allen G, Brauny M, Linh LTK, Pallerla SR, Huyen TTT, et al. Molecular surveillance and temporal monitoring of malaria parasites in focal Vietnamese provinces. Malar J. 2020;19:458. https://doi.org/10.1186/s12936-020-03561-6.
5. World Health Organization (WHO). World Malaria Report 2016. 2016. https://www.who.int/publications/i/item/9789241511711. Accessed 11 Apr 2021.
6. World Health Organization (WHO). Malaria. 2021. https://www.who.int/news-room/fact-sheets/detail/malaria. Accessed 18 Jan 2021.
7. Moncayo AC, Fernandez Z, Ortiz D, Diallo M, Sall A, Hartman S, et al. Dengue emergence and adaptation to peridomestic mosquitoes. Emerg Infect Dis. 2004;10:1790–6.
8. Brady OJ, Gething PW, Bhatt S, Messina JP, Brownstein JS, Hoen AG, et al. Refining the global spatial limits of dengue virus transmission by evidence-based consensus. PLoS Negl Trop Dis. 2012;6: e1760.
9. World Health Organization (WHO). Global strategy for dengue prevention and control, 2012–2020. https://www.who.int/denguecontrol/9789241504034/en/. Accessed 16 May 2021.
10. Van Dung N. Checklist of the known species of Culicidae found in Vietnam. Hoi Nghi Khoa Hoc Toan Quoc Ve Sinh Thai va Tai Nguyen Sinh Vat Lan. 2021, http://iebr.ac.vn/database/HNTQ6/504.pdf. Accessed 18 Jan 2021.
11. Tandina F, Doumbo O, Yaro AS, Traoré SF, Parola P, Robert V. Mosquitoes (Diptera: Culicidae) and mosquito-borne diseases in Mali, West Africa. Parasit Vectors. 2018;11:467.
12. Nadler SA, DE León GPP. Integrating molecular and morphological approaches for characterizing parasite cryptic species: implications for parasitology. Parasitology. 2011;138:1688–709.
13. Green CA, Gass RF, Munstermann LE, Baimai V. Population-genetic evidence for two species in *Anopheles minimus* in Thailand. Med Vet Entomol. 1990;4:25–34.
14. Lauri A, Mariani PO. Potentials and limitations of molecular diagnostic methods in food safety. Genes Nutr. 2009;4:1–12.
15. Hebert PDN, Cywinska A, Ball SL, De Waard JR. Biological identifications through DNA barcodes. Proc Biol Sci. 2003;270:313–21.
16. Vega-Rúa A, Pagès N, Fontaine A, Nuccio C, Hery L, Goindin D, et al. Improvement of mosquito identification by MALDI-TOF MS biotyping using protein signatures from two body parts. Parasit Vectors. 2018;11:574.
17. Laroche M, Jean-Michel Bérenger JM, Pascal Delaunay P, Didier Raoult D, Parola P, et al. Medical entomology: a reemerging field of research to better understand vector-borne infectious diseases. Clin Infect Dis. 2017;65:S30-8.
18. Sevestre J, Diarra AZ, Laroche M, Almeras L, Parola P. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: an emerging tool for studying the vectors of human infectious diseases. Future Microbiol. 2021;16:323–40.
19. Yssouf A, Socolovschi C, Flaudrops C, Sokhna CS, Raoult D, Parola P, et al. Matrix-assisted laser desorption ionization—time of flight mass spectrometry: an emerging tool for the rapid identification of mosquito vectors. PLoS ONE. 2013. https://doi.org/10.1371/journal.pone.0072380.
20. Diarra AZ, Laroche M, Berger F, Parola P. Use of MALDI-TOF MS for the identification of chad mosquitoes and the origin of their blood meal. Am J Trop Med Hyg. 2019;100:47–53.
21. Yssouf A, Almeras L, Raoult D, Parola P. Emerging tools for identification of arthropod vectors. Future Microbiol. 2016;11:549–66.
22. Diarra AZ, Almeras L, Laroche M, Doumbo O, Raoult D, Parola P, et al. Molecular and MALDI-TOF identification of ticks and tick-associated bacteria in Mali. PLoS Negl Trop Dis. 2017;11:e0005762.
23. Lawrence AL, Jana Batovska J, Webb CE, Šlapeta J, Parola P, Laroche M, et al. Accurate identification of Australian mosquitoes using protein profiling. Parasitology. 2019;146:462–71.
24. Benkacimi L, Gazelle G, El Hamzaoui B, Bérenger JM, Parola P, Laroche M. MALDI-TOF MS identification of Cimex lectularius and *Cimex hemipterus* bedbugs. Infect Genet Evol. 2020;85:104536. https://doi.org/10.1016/j.meegid.2020.104536.
25. Ouarti B, Laroche M, Righi S, Meguini MN, Benakhla A, Raoult D, et al. Development of MALDI-TOF mass spectrometry for the identification of lice isolated from farm animals. Parasite. 2020. https://doi.org/10.1051/parasite/2020026.
26. Favrot C. Polymerase chain reaction: advantages and drawbacks. In: 3. Congresso Latinoamericano de Dermatologia Veterinaria [Third Latin American Congress of Veterinary Dermatology], 26–27 Nov 2015; Buenos Aires. https://www.zora.uzh.ch/id/eprint/116536. Accessed 18 Jan 2021.
27. National Institute of Malariology, Parasitology and Entomology (NIMPE) Vietnam. Bang Dinh Loai muoi Anophelinae o Vietnam. 2006. http://nimpe.vn/TinChiTiet.aspx?id=33&cat=4. Accessed 21 Dec 2020.
28. Rattanarithikul R, Harrison BA, Harbach RE, Panthusiri P, Coleman RE. Illustrated keys to the mosquitoes of Thailand. IV. Anopheles. Southeast Asian J Trop Med Public Health. 2006;37:1–128.
29. Reuben R, Tewari SC, Hiriyan J, Akiyama J. Illustrated keys to species of *Culex* (*Culex*) associated with Japanese encephalitis in Southeast Asia (Diptera: Culicidae). Mosquito Systematics. 1994;26:75–96.
30. Rueda LM. Pictorial keys for the identification of mosquitoes (Diptera: Culicidae) associated with dengue virus transmission. Zootaxa. 2004;586:1–60.
31. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3:294–9.
32. Kambhampati S. A phylogeny of cockroaches and related insects based on DNA sequence of mitochondrial ribosomal RNA genes. Proc Natl Acad Sci USA. 1995;92:017–20.
33. Smith JL, Fonseca DM. Rapid assays for identification of members of the *Culex* (*Culex*) *pipiens* complex, their hybrids, and other sibling species (Diptera: culicidae). Am J Trop Med Hyg. 2004;70:339–45.
34. Collins FH, Paskewitz SM. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. Insect Mol Biol. 1996;5:1–9.
35. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33:1870–4.
36. Nebbak A, Willcox AC, Bitam I, Raoult D, Parola P, Almeras L. Standardization of sample homogenization for mosquito identification using an innovative proteomic tool based on protein profiling. Proteomics. 2016;16:3148–60.
37. Yssouf A, Parola P, Lindström A, Lilja T, Berenger JM, Raoult D, et al. Identification of European mosquito species by MALDI-TOF MS. Parasitol Res. 2014;113:2375–8.
38. Nabet C, Kone AK, Dia AK, Sylla M, Gautier M, Yattara M, et al. New assessment of *Anopheles* vector species identification using MALDI-TOF MS. Malar J. 2021;20:33.
39. Rakotonirina A, Pol M, Kainiu M, Barsac E, Tutagata J, Kilama S, et al. MALDI-TOF MS: optimization for future uses in entomological surveillance and identification of mosquitoes from New Caledonia. Parasit Vectors. 2020;13:359.
40. Do MC, Beebe NW, Nguyen TVV, Nguyen XT, Le NA, Cooper RD, et al. Vectors and malaria transmission in deforested, rural communities in north-central Vietnam. Malar J. 2010;9:259.
41. Garros C, Van Nguyen C, Trung HD, Bortel WV, Coosemans M, Manguin S. Distribution of *Anopheles* in Vietnam, with particular attention to malaria vectors of the *Anopheles minimus* complex. Malar J. 2008;7:11.
42. Ngo CT, Dubois G, Sinou V, Parzy D, Le HQ, Harbach RE, Manguin S. Diversity of *Anopheles* mosquitoes in Binh Phuoc and Dak Nong provinces of Vietnam and their relation to disease. Parasit Vectors. 2014;7:316.
43. Marasri N, Overgaard HJ, Sumarnrote A, Thanispong K, Corbel V, Chareonviriyaphap T. Abundance and distribution of *Anopheles* mosquitoes in a malaria endemic area along the Thai–Lao border. J Vector Ecol. 2017;42:325–34.

44. Vantaux A, Samreth R, Piv E, Siv S, Taylor WR, Ménard D, et al. Contribution to malaria transmission of symptomatic and asymptomatic parasite carriers in Cambodia. J Infect Dis. 2018;217:1561–8.
45. World Health Organization (WHO). Dengue situation update 533, 2 January 2018. 2018. https://reliefweb.int/report/viet-nam/dengue-situation-update-533-2-january-2018. Accessed 17 Jan 2021.
46. Quy N. Dengue fever outbreak kills 50 across Vietnam. *VnExpress International*. 31 Oct 2019. https://e.vnexpress.net/news/news/dengue-fever-outbreak-kills-50-across-vietnam-4005827.html. Accessed 17 Jan 2021.
47. Dang TT, Pham HM, Bui VH, Le VD. Whole genome sequencing and genetic variations in several dengue virus type 1 strains from unusual dengue epidemic of 2017 in Vietnam. Virol J. 2020;17:7.
48. Quyen NTH, Kien DTH, Rabaa M, Van Vinh Chau N, Wills B, Simmons CP, et al. Chikungunya and zika virus cases detected against a backdrop of endemic dengue transmission in Vietnam. Am J Trop Med Hyg. 2017;97:146–50.
49. Nguyen TY, Duffy MR, Nguyen MH, Nguyen TH, Fischer M, Hills SL. Surveillance for Japanese encephalitis in Vietnam, 1998–2007. Am J Trop Med Hyg. 2010;83:816–9.
50. Ngan D. Nang nong, canh giac cao voi benh viem nao Nhat Ban. HQ Online. 2020. https://haiquanonline.com.vn/nang-nong-canh-giac-cao-voi-benh-viem-nao-nhat-ban-127028-127028.html. Accessed 17 Jan 2021.
51. Shaikevich EV, Vinogradova EB, Bouattour A, de Almeida APG. Genetic diversity of *Culex pipiens* mosquitoes in distinct populations from Europe: contribution of *Culex quinquefasciatus* in Mediterranean populations. Parasit Vectors. 2016;9:47.
52. Batovska J, Blacket MJ, Brown K, Lynch SE. Molecular identification of mosquitoes (Diptera: Culicidae) in southeastern Australia. Ecol Evol. 2016;6:3001–11.
53. Tandina F, Niaré S, Laroche M, Doumbo OK, Raoult D, Parola P, et al. Using MALDI-TOF MS to identify mosquitoes collected in Mali and their blood meals. Parasitology. 2018;145:1170–82.
54. Nebbak A, El Hamzaoui B, Berenger J-M, Bitam I, Raoult D, Almeras L, et al. Comparative analysis of storage conditions and homogenization methods for tick and flea species for identification by MALDI-TOF MS. Med Vet Entomol. 2017;31:438–48.
55. Harbach RE, Garros C, Manh CN, Manguin S. Formal taxonomy of species C of the *Anopheles minimus* sibling species complex (Diptera: Culicidae). Zootaxa. 2007;1654:1. https://doi.org/10.11646/zootaxa.1654.1.3.
56. Harbach RE, Parkin E, Chen B, Butlin RK. *Anopheles* (Cellia) *minimus* theobald (Diptera: Culicidae): neotype designation, characterization, and systematics. Proc Entomol Soc Wash. 2006;108:198–209.
57. Niare S, Tandina F, Davous B, Raoult D, Parola P, Lionel Almeras L, et al. Accurate identification of *Anopheles gambiae* Giles trophic preferences by MALDI-TOF MS. Infect Genet Evol. 2018;63:410–9.

ARTICLE II

Morphological, molecular and MALDI-TOF MS identification of ticks and tick-associated pathogens in Vietnam

Ly Na Huynh, Adama Zan Diarra, Quang Luan Pham, Nhiem Le-Viet, Jean-Michel Berenger, Van Hoang Ho, Xuan Quang Nguyen, Philippe Parola*
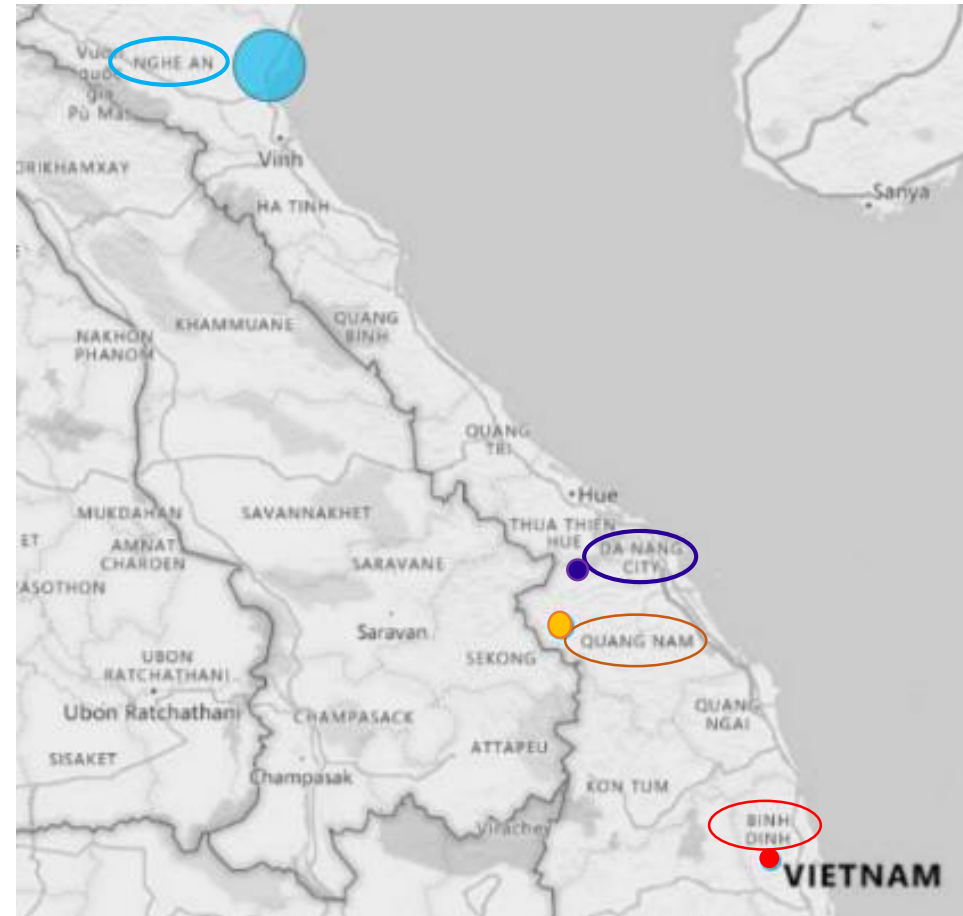
Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been described as a promising and dependable tool for identifying arthropods, including the identification of alcohol-preserved ticks using extracted leg protein spectra. In this study, the legs of 361 ticks were collected from wild and domestic animals in September 2010, between April and September 2018 in Vietnam, including 251 *Rhiphicephalus sanguineus* s.l, 99 *Rhipicephalus* (*Boophilus*) *microplus*, two *Amblyomma varanensis*, seven *Dermacentor auratus*, one *Dermacentor compactus*, and one *Amblyomma* sp. All these species had been reported in Vietnam. However, our study is the first to observe *Am. varanensis, Amblyomma* sp. on pangolins from Vietnam. Molecular techniques were used to confirm our morphological identification of tick species by amplifying a portion sequence of a 465-bp fragment 16S rRNA gene. Unfortunately, we were unable to obtain sequences for all specimens that had been preserved in alcohol for more than ten years (i.e., *Am. varanensis*, *Amblyomma sp.*, *D. auratus*, and *D. compactus*) with the *16S* and *12S* rDNA genes. This could be because the alcohol was not completely removed during extraction, or because the ticks contained blood from their host, which consists several factors that can inhibit the PCR reaction, as previously reported. Spectral analysis showed intra-species reproducibility and inter-species specificity and the spectra of 329 (91%) specimens were of excellent quality. The blind test of 310 spectra remaining after updating the database with 19 spectra revealed that all were correctly identified with log score values (LSV) ranging from 1.7 to 2.396 with a mean of 1.982 ± 0.142 and a median of 1.971. Interestingly, specimens that could not be identified using molecular biology were identified using MALDI-TOF MS. This demonstrates that the tool is reliable and accurate for tick identification. The microorganisms identified in this study and confirmed by sequencing are Anaplasmataceae (*A. phagocytophilum*, *A. marginale*, *A.*

platys, and *E. rustica*), which are known aetiologies of zoonotic diseases and the Piroplasmida family (*B. vogeli*, *T. sinensis,* and *T. orientalis*) was mainly known as the potential zoonotic pathogens. Co-infection by *A. phagocytophilum* and *T. sinensis* was found in one *Rh.* (*B*) *microplus*. These findings suggested that ticks and tick-associated pathogens are likely to pose challenges to public and veterinary health in Vietnam.

624 ticks were in 70% alcohol

Sampling Collection sites:
Nghe An, Da Nang, Quang Nam, Binh Dinh
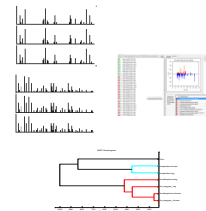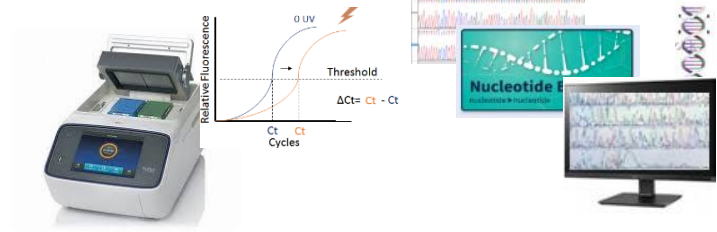
Host collection

Morphological ID

Incubated at 56°C

Over night

DNA extraction

O.N at 37°C for 2 times:
- Tick without H
- Tick with 40µL HLGH

MALDI-TOF MS analysis

qPCR

Sequnecing

Molecular analysis

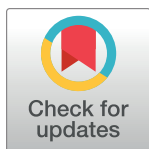**Location of study sites in Vietnam**

30

RESEARCH ARTICLE

Morphological, molecular and MALDI-TOF MS identification of ticks and tick-associated pathogens in Vietnam

Ly Na Huynh[1,2,3], Adama Zan Diarra[1,2], Quang Luan Pham[3], Nhiem Le-Viet[4], Jean-Michel Berenger[1,2], Van Hoang Ho[3], Xuan Quang Nguyen[3], Philippe Parola[1,2] *

1 Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France, **2** IHU-Méditerranée Infection, Marseille, France, **3** Institute of Malariology, Parasitology and Entomology, Quy Nhon (IMPE-QN), Vietnam, **4** School of Medicine and Pharmacy, The University of Da Nang (UD), Da Nang, Vietnam

* philippe.parola@univ-amu.fr

Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been reported as a promising and reliable tool for arthropod identification, including the identification of alcohol-preserved ticks based on extracted leg protein spectra. In this study, the legs of 361 ticks collected in Vietnam, including 251 *Rhiphicephalus sanguineus* s.l, 99 *Rhipicephalus* (*Boophilus*) *microplus*, two *Amblyomma varanensis*, seven *Dermacentor auratus*, one *Dermacentor compactus* and one *Amblyomma* sp. were submitted for MALDI-TOF MS analyses. Spectral analysis showed intra-species reproducibility and inter-species specificity and the spectra of 329 (91%) specimens were of excellent quality. The blind test of 310 spectra remaining after updating the database with 19 spectra revealed that all were correctly identified with log score values (LSV) ranging from 1.7 to 2.396 with a mean of 1.982 ± 0.142 and a median of 1.971. The DNA of several microorganisms including *Anaplasma platys*, *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Ehrlichia rustica*, *Babesia vogeli*, *Theileria sinensis*, and *Theileria orientalis* were detected in 25 ticks. Co-infection by *A. phagocytophilum* and *T. sinensis* was found in one *Rh.* (*B*) *microplus*.

Author summary

Ticks are one of the important vectors and reservoirs of multiple pathogens infecting humans and animals such as bacteria, protozoans, viruses, and helminths. Nevertheless, studies on ticks and tick-borne infections remain limited in Vietnam. That said, serological and molecular evidence of tick infections in animals and humans have been reported on several occasions in Vietnam and Southeast Asia in recent decades. The identification of ticks and tick-associated diseases has an important role to play in epidemiological investigation and in assessing the risks of disease transmission to humans and animals. Recently, MALDI-TOF MS has been used as an innovative tool for the rapid and accurate identification of alcohol-preserved ticks based on proteins from extracted legs. This procedure represents a time-cost saving and does not require expert knowledge. This goal of

31

this study was to assess the efficiency and reliability of MALDI-TOF MS for the identification of alcohol-preserved ticks collected in Vietnam and to determine the presence of their relative pathogens. Our study revealed 97% correspondence between morphological and MALDI-TOF MS identification. The detected microorganisms that were confirmed by sequencing belonged to the Anaplasmataceae and Piroplasmida families. These findings suggested that ticks and tick-associated pathogens are likely to pose challenges to public and veterinary health in Vietnam.

Introduction

Ticks have been incriminated as the second most important vectors of human and animal infectious pathogens in the world after mosquitoes [1] and are able to transmit a wide range of pathogens, including bacteria, protozoans, viruses, and helminths [2]. In Southeast Asia (SEA), there are 104 known tick species, representing 12 genera, which is approximately 12% of all recognised and classified species [3]. Among them, *Rhipicephalus sanguineus* sensu lato (s.l.) are the most common ticks that parasitise dogs in SEA. These ticks are the ectoparasite vectors of bacterial and protozoal pathogens that can be transmitted to animals [4] and humans [5]. *Rhipicephalus* (*Boophilus*) *microplus* is an important vector of livestock pathogens [6]. *Amblyomma* (formerly *Aponomma*) *varanensis*, *Dermacentor auratus*, and *Dermacentor compactus* may act as vectors of infectious agents (e.g. *Rickettsia* spp., *Anaplasma* spp., *Ehrlichia* spp., *Borrelia* spp., *Babesia* spp. and *Theileria* spp.) to humans, and to domestic and wild animals in Malaysia, Laos, Thailand, and Vietnam [7–10].

In Vietnam, the agricultural sector makes up one-third of the developing nation's economy [11], and livestock represents the second biggest contribution to household incomes after crop growing [12]. Despite the perceived food and economic benefits of livestock production, the country is potentially faced with challenges such as the emergence and re-emergence of zoonotic diseases, which can cause huge losses [13, 14]. One such example is the risk of infectious diseases spreading through the large number of dogs that are illegally imported into Vietnam from neighbouring countries for food consumption without any veterinary controls [15, 16]. In 2014, an outbreak of oriental theileriosis, which causes abortion and death, in imported cattle from Australia to Vietnam was associated with *Theileria orientalis* [17]. The serological detection of both *Babesia bovis* and *Babesia bigemina* parasite species transmitted by ticks has also been reported in cattle imported from Thailand [18].

Limited data is available on ticks and tick-associated pathogens in Vietnam. Nevertheless, 48 species of nine tick genera have been reported by Kolonin [19] and recently two new species of ticks of the genus *Dermacentor* (*Dermacentor limbooliati* and *Dermacentor filippovae*) have been described by Apanaskevich [9, 20]. Also in Vietnam, some tick-borne microorganisms have been reported in ticks and animals [19, 21–23], more precisely in *Hepatozoon canis*, *Ehrlichia canis*, and *Babesia vogeli* ticks [24].

In recent years, several studies have focused on acarology in Vietnam [4, 10, 25]. The correct identification of ticks is a crucial step in distinguishing tick vectors from non-vectors. The lack of reference data and standard taxonomic keys specific to Vietnamese tick species makes the morphological identification of Vietnamese ticks difficult or almost impossible. The morphological identification of tick species therefore remains a challenge for Vietnamese researchers [19]. Molecular tools have been used to overcome the limitations of morphological identification [26]. However, there are several drawbacks to these tools, which are time-consuming, expensive, and require primer-specific targeting [27–29].

32

Recently, the MALDI-TOF MS method has been proposed as an alternative and innovative tool to overcome the limitations of the above two methods in arthropod identification [30]. Since then, studies in several laboratories have demonstrated that MALDI-TOF MS is a remarkably robust tool for identifying many species of arthropod vectors and non-vectors [30]. The aim of this study was to identify tick species collected from domestic and wild animals in Vietnam and their associated pathogens using morphological, MALDI-TOF MS and molecular tools.

Materials and methods

Ethics statement

Ethical approval was obtained from the Institute of Malariology, Parasitology, and Entomology, Quy Nhon (IMPE-QN) on behalf of the Vietnamese Ministry of Health (approval no: 401/VSR-CT-2010, 333/CT-VSR-2018). Permission was obtained from the communal authorities for wild animals that were not listed in the Red Data Book of Vietnam, and agreement was obtained from the owners of cows, goats, and dogs.

Tick collection and morphological identification

Ticks were collected in four provinces: Quynh Luu (19°13' N; 105°60' E) District, Nghe An Province; Nam Giang (15°65' N; 107°50' E) District, Quang Nam Province; Van Canh (13°37' N; 108°59' E) District, Binh Dinh Province; and Khanh Vinh (12°16' N; 108°53' E) District, Khanh Hoa Province in Vietnam in September 2010, between April and September 2018. The map of Vietnam showing the collection sites was made with QGIS version 3.10 and the Vietnamese layers were downloaded from DIVA-GIS at the following link: https://www.diva-gis.org/datadown (Fig 1A). All engorged and non-engorged ticks were collected from the skin of domestic animals (cows, goats, and dogs) and wild animals (pangolins, wild pigs) using forceps. Ticks from wild animals were collected in a collaborative manner by rangers and trained care personnel from the Wildlife Rescue, Conservation and Development Center. Ticks were morphologically identified first at species level using dichotomous keys [9, 31] by an entomological team from the Institute of Malariology, Parasitology and Entomology, Quy Nhon,
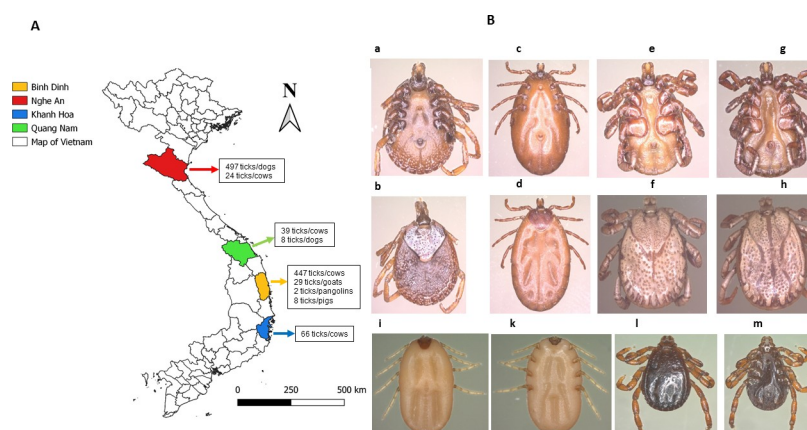
Fig 1. Map of Vietnam showing tick collection sites realised with QGIS version 3.10, the layers have been uploaded to the DIVA-GIS website: **https://www.diva-gis.org/datadown** (A); Morphologically, the 70% alcohol tick-preserved species were collected in Vietnam over a period of 10 years: *Amblyomma varanensis* [♀: **a, b**]; *Amblyomma* sp. [♀: **c, d**]; *Dermacentor auratus* [♂: **e, f**]; *Dermacentor compactus* [♂: **g, h**]; and approximately 2 years: *Rhipicephalus* (B) *microplus* [♀: **I, k**]; *Rhipicephalus sanguineus* s.l [♂: **l, m**] **(B)**.

33

Vietnam (IMPE-QN). Ticks from the same host were counted and placed in the same tube containing 70% v/v alcohol, before being sent to the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection in Marseille, France for MALDI-TOF MS and molecular analysis. In Marseille, the morphological identification of ticks was verified by two specialists in morphological identification of ticks using a magnifying glass (Zeiss Axio Zoom.V16, Zeiss, Marly le Roi, France) and dichotomous keys. Morphological identification was carried out only if all discriminating characters had been observed.

Tick dissection and sample preparation

Ticks were individually removed from the alcohol and were rinsed and dissected with a sterile surgical blade, as previously described [32]. The four legs of each tick and the half part without legs were submitted for MALDI-TOF MS and molecular biology analysis, respectively. The remaining parts with legs were frozen and stored as samples for any further research.

DNA extraction and molecular identification of ticks

DNA from each half-tick or legs (for ticks from which we did not obtain sequences with half-tick DNA) was individually extracted using an EZ1 DNA tissue kit (Qiagen), according to the manufacturer's recommendations, as previously described [33]. DNA was monitored with Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and either immediately used or stored at -20°C until use.

DNA from ticks was submitted to standard PCR in an automated DNA thermal cycle to amplify a 465-base pair (bp) fragment of the mitochondrial *16S* DNA gene, as described previously [34]. The *12S* tick gene, amplifying about 405-bp of the mitochondrial DNA fragment, was used for all specimens for which we did not have a sequence with the *16S* gene. DNA from *Rh. sanguineus* s.l., reared in our laboratory, was used as a positive control. Purified PCR products were sequenced as previously described [34]. The obtained sequences were assembled and analysed using the ChromasPro software (version 1.7.7) (Technelysium Pty. Ltd., Tewantin, Australia), and were then blasted against the reference sequences available in GenBank (http://blast.ncbi.nlm.nih.gov/).

MALDI-TOF MS analysis

Sample preparation. The four legs of each tick were first put into an Eppendorf tube and dried overnight at 37°C and then put into an Eppendorf tube with 40 μL of high-performance liquid chromatography (HPLC) grade water and incubated overnight at 37°C. The legs were then crushed in a mix of 20 μL of 70% (v/v) formic acid (Sigma) and 20 μL of 50% (v/v) acetonitrile (Fluka, Buchs, Switzerland), with glass beads (Sigma, Lyon, France), as described previously [35]. The crushed legs were centrifuged and 1 μL of the supernatant of each sample was deposited in quadruplicate onto a MALDI-TOF MS steel plate (Bruker Daltonics, Wissembourg, France). After drying at room temperature, 1μL of matrix solution composed of a saturated solution of α-cyano-4-hydroxycynnamic acid (Sigma, Lyon, France), 50% acetonitrile (v/v), 2. 5% trifluoroacetic acid (v/v) (Aldrich, Dorset, United Kingdom), and high performance liquid chromatography (HPLC) grade water was added [36]. The target plate was air-dried one more at room temperature before being introduced into the Microflex LT MALDI-TOF Mass Spectrometer (Bruker Daltonics, Germany) for analysis. The quality of the matrix, sample loading, and performance of the MALDI-TOF MS device were controlled using the legs of a *Rh. sanguineus* s.l. reared in our laboratory as a positive control.

MALDI-TOF MS parameters, spectral analysis and reference database creation. The spectral profiles obtained from the tick legs were visualised using a Microflex LT MALDI-TOF

34

mass spectrometer with FlexControl software (version 3.3, Bruker Daltonics). The setting parameters of the MALDI-TOF MS apparatus were identical to those previously used [32].

The FlexAnalysis v.3.3 software was used to evaluate spectral quality (smoothing, baseline subtraction, peak intensities). MS spectra reproducibility was assessed by comparing the average spectral profiles (MSP, main spectrum profile) obtained from the four spots of each tick leg, according to species, using MALDI-Biotyper v3.0 software (Bruker Daltonics) [37]. MS spectra reproducibility and specificity were assessed based on a principal component analysis (PCA) and cluster analysis (MSP dendrogram). PCA was performed using ClinProTools v2.2 with the manufacturer's default settings. Cluster analysis was performed based on a comparison of the MSP given by MALDI-Biotyper v3.0. software with clustering according to protein mass profile (i.e., their mass signals and intensities) [37].

Based on the morphological identification, eight and seven reference spectra of *Rh. sanguineus* and *Rh. (B) microplus*, respectively, were added to our MALDI-TOF MS database. However, two, one, and one spectra of *D. auratus*, *Am. varanensis*, *D. compactus*, respectively, which were only identified morphologically by three tick identification specialists, were also added to our MALDI-TOF MS database. To create a database, reference spectra (MSP, Main Spectrum Profile) were created by combining the results of spectra from specimens of each species using the automated function of the MALDI-Biotyper v3.0 software (Bruker Daltonics). MSPs were created based on an unbiased algorithm using peak position, intensity, and frequency data [38]. Four tick species that could not be identified by molecular biology were temporarily added into the MS reference database to identify the remaining specimens from the same species.

Blind test for tick identification. A blind test was performed with the remaining tick specimens not included in our MALDI-TOF MS database after the database had been upgraded with 19 MS spectra from specimens of the five tick species to determine their identification. The reliability of tick species identification was estimated using the log score values (LSVs) obtained from the MALDI-Biotyper software, which ranged from 0 to 3. These LSVs correspond to the degree of similarity between the MS reference spectra in the database and those submitted to blind tests. An LSV was obtained for each spectrum of the samples tested. According to one previous study [37], an LSV of at least 1.8 should be obtained to be considered reliable for species identification.

Detection of microorganisms. Quantitative PCR (qPCR) was performed for screening microorganisms using specific primers and probes targeting Anaplasmataceae, Piroplasmida, *Borrelia* spp., *Bartonella* spp., *Coxiella burnetii*, and *Rickettsia* spp. PCR reactions were performed according to the manufacturer's instructions, using a CFX96 Touch detection system (Bio-Rad). qPCR amplification was performed using the thermal profile described previously [39]. The DNA of *Rickettsia montanensis*, *Bartonella elizabethae*, *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Borrelia crocidurae*, and *Babesia vogeli* were used as a positive control and DNA from *Rh. sanguineus* s.l from our laboratory, which were free of bacteria, were used as negative controls. The samples were considered to be positive when the cycle threshold (Ct) was strictly less than 36 [40].

All samples that were positive following qPCR were submitted to standard PCR and sequencing to identify the microorganism species. For the *Rickettsia* sp. positive sample, we first used a primer targeting a 630-bp fragment of the *OmpA* gene [35] and then another targeting a 401-bp fragment of the *gltA* gene [33]. Samples which were Anaplasmataceae positive following qPCR were subjected to amplifying and sequencing of a 520-bp fragment of the *23S* rRNA gene [33]. Samples which were Piroplasmidae positive following qPCR were subjected to amplifying and sequencing of a 969-bp fragment of the *18S* rRNA [41]. Samples which were *Borrelia* sp. positive following qPCR was subjected to amplifying and sequencing of a 344-bp

35

Table 1. Target amplified and used for qPCR and standard PCR.

Microorganisms	Targeted sequence	Primers (5'-3') and Probes (Used for qPCR Screening or Sequencing)	References
Anaplasmataceae	23S	f_TGACAGCGTACCTTTTGCAT r_GTAACAGGTTCGGTCCTCCA p_6FAM-GGATTAGACCCGAAACCAAG	[108]
	23S (520-bp)	f_ATAAGCTGCGGGGAATTGTC r_TGCAAAAGGTACGCTGTCAC	
Piroplasmida	5.8S	f_AYYKTYAGCGRTGGATGTC r_TCGCAGRAGTCTKCAAGTC p_FAM-TTYGCTGCGTCCTTCATCGTTGT-MGB	[39]
	18S (969-bp)	f1_GCGAATGGCTCATTAIAACA f4_CACATCTAAGGAAGGCAGCA f3_GTAGGGTATTGGCCTACCG* r4_AGGACTACGACGGTATCTGA*	
***Rickettsia* spp.**	gltA(RKND03)	f_GTGAATGAAAGATTACACTATTTAT r_GTATCTTAGCAATCATTCTAATAGC p_6FAM-CTATTATGCTTGCGGCTGTCGGTTC	[109]
	gltA (401-bp)	f_ATGACCAATGAAAATAATAAT r_CTTATACTCTCTATGTACA	[110]
	OmpA (630-bp)	70_ATGGCGAATATTTCTCCAAAA 701_GTTCCGTTAATGGCAGCATCT 180_GCAGCGATAATGCTGAGTA*	[1]
***Borrelia* spp.**	ITS4	f_GGCTTCGGGTCTACCACATCTA r_CCGGGAGGGGAGTGAAATAG p_TGCAAAAGGCACGCCATCACC	[111]
	flaB (344-bp)	f_TGGTATGGGAGTTTCTGG r_TAAGCTGACTAATACTAATTACCC	
***Bartonella* spp.**	ITS2	f_GATGCCGGGGAAGGTTTTC r_GCCTGGGAGGACTTGAACCT p_GCGCGCGCTTGATAAGCGTG	[112]
Coxiellia burnetii	IS30A	f_CGCTGACCTACAGAAATATGTCC r_GGGGTAAGTAAATAATACCTTCTGG p_CATGAAGCGATTTATCAATACGTGTATG	[113]

Abbreviation

*, used for sequencing only.

fragment of the *flaB* gene [42]. The primers and probes used in this study are listed in Table 1. The obtained sequences were assembled and analysed using the ChromasPro software (version 1.7.7) (Technelysium Pty. Ltd., Tewantin, Australia), and were then blasted against the reference sequences available in GenBank (http://blast.ncbi.nlm.nih.gov/). The method used for phylogenetic tree analysis was the neighbour-joining (NJ) method with 1,000 replicates. DNA sequences were aligned using MEGA software version 7.0 (https://www.megasoftware.net/). The various statistical analyses were performed using R software version 3.4 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) and ggplot packages were used to perform the graphics.

Results

Tick collection and morphological identification

A total of 1120 ticks including 334 (30%) engorged ticks were collected in four provinces of Vietnam: Nghe An, Quang Nam, Binh Dinh, and Khanh Hoa. Morphologically, ticks were identified as belonging to six species (Fig 1A), including 935 (83.5%) *Rh. sanguineus* s.l. collected from dogs, 174 (15.5%) *Rh.* (*B*) *microplus*) from cows and goats, seven (0.6%) *D. auratus*

36

Table 2. The number of tick species used for MALDI-TOF MS analysis, creation of the MS reference spectra creation, and molecular biology confirmation.

Morphological identification	Number submitted for molecular ID*	Molecular ID* (%identity; GenBank accession number)	Number of good spectra/ tested	Number of spectra added to DB$	MADI-TOF MS ID* (number identified)	LSVs & [Low-High]
Rhipicephalus sanguineus s.l	8	*Rh. sanguineus* s.l (99.75–100%; MG651947, MG793434, KX632154)	241/251	8	*Rh. sanguineus* s.l (233)	[1.7–2.351]
Rhipicephalus (B) microplus	7	*Rh.* (*B*) *microplus* (100%; MN880401, MT462222, EU918187)	78/99	7	*Rh.* (*B*) *microplus* (71)	[1.705–2.346]
Amblyomma varanensis	1	not identified	1/2	1	NA	NA
Amblyomma sp.	1	not identified	1/1	0	*Am.varanensis* (1)	1.857
Dermacentor auratus	7	not identified	7/7	2	*D. auratus* (5)	[1.949–2.396]
Dermacentor compactus	1	not identified	1/1	1	NA	NA
Total	**25**		**329/361**	**19**	**310**	

*Identification

& Range of log score values

$ Database.

https://doi.org/10.1371/journal.pntd.0009813.t002

from pangolins, two (0.2%) *Am. varanensis* from wild pigs, and one (0.1%) *D. compactus* and one (0.1%) *Amblyomma* sp. from a pangolin (Table 2). *Rhipicephalus sanguineus* s.l. and *Rh. (B) microplus* were collected between April and September 2018. The other ticks were collected in September 2010. The different specimens that could not be identified by molecular biology are shown in the pictures in Fig 1B that we took using a magnifying glass (Zeiss Axio Zoom. V16, Zeiss, Marly le Roi, France).

Molecular identification of ticks

To confirm our morphological identification, 25 tick specimens were submitted to molecular analysis using the *16S* rDNA gene, including eight specimens of *Rh. sanguineus* s.l., seven *Rh. (B) microplus*, seven *D. auratus*, one *Am. varanensis*, one *D. compactus* and one *Amblyomma*
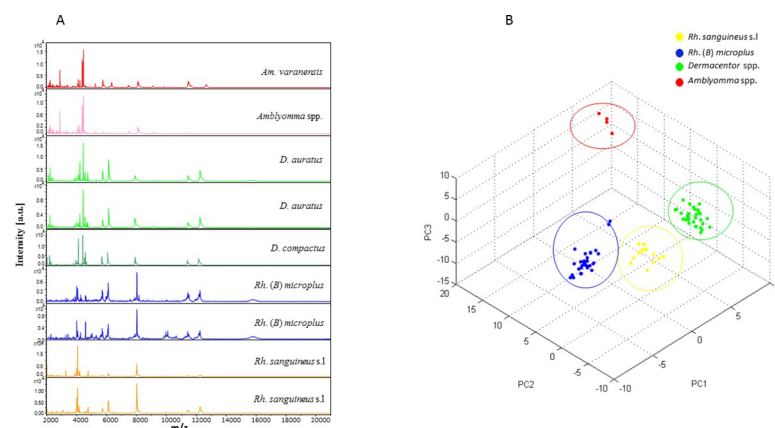

Fig 2. Comparison of MALDI-TOF MS spectra from the legs of six tick species collected in Vietnam. The MS spectra revealed intra-species reproducibility and inter-species specificity (A); The MS spectra were compared by Principal Component Analysis (B); a.u., arbitrary units; m/z, mass-to-charge ratio.

https://doi.org/10.1371/journal.pntd.0009813.g002

37

sp. Sequences were obtained only for the specimens of *Rh. sanguineus* s.l. and *Rh.* (*B*) *microplus*. BLAST analysis indicated that obtained sequences from *Rh. sanguineus* s.l. were 99.75 to 100% identical to the corresponding sequences of *Rh. sanguineus* s.l. (Genbank: MG651947, MG793434, KX632154) and those obtained from *Rh.* (*B*) *microplus* were 100% identical to the corresponding sequences of *Rh.* (*B*) *microplus* (Genbank: MN880401, MT462222, EU918187). Unfortunately, for the specimens morphologically identified as *D. auratus*, *Am. varanensis*, *Amblyomma* sp. and *D. compactus*, we were unable to amplify any DNA from the half-tick or legs of these tick species with PCR targeting part of the two genes (*16S* and *12S* rDNA), despite the fact that the nanodrop had indicated that the amount of DNA contained in these samples was 7.8 to 19.4 ng/μl.

MS reference spectra analysis

The legs of 361 specimens, including 251 morphologically identified as *Rh. sanguineus* s.l., 99 *Rh.* (*B*) *microplus*, seven *D. auratus*, two *Am. varanensis*, one *Amblyomma* sp. and one *D. compactus* were randomly selected and subjected to MALDI-TOF MS analysis. Visualisation of MS spectra from all specimens using FlexAnalysis v.3.3 software showed that 91% (329) of specimens had excellent quality spectra (peak intensity > 3,000 a.u., no background noise and baseline subtraction correct) (Figs 2A and S1 and Table 2). The MS spectra of different specimens showed intra-species reproducibility and inter-species specificity, as confirmed by PCA (Figs 2B and 3B) and dendrogram (Fig 3A) analysis. PCA and dendrogram analysis showed that all specimens of the same species were grouped together or were on the same branches. Additionally, at the genus level, all specimens from the same genus were also gathered in the same part of dendrogram (Fig 3A).

MALDI-TOF MS tick identification by blind test

The 310 MS remaining spectra of excellent quality, including 233 *Rh. sanguineus* s.l, 71 *Rh.* (*B*) *microplus*, five *D. auratus* and one *Amblyomma* sp. were queried against our reference spectra database upgraded with eight *Rh. sanguineus* s.l. and seven *Rh.* (*B*) *microplu*s which were morphologically and molecularly identified, and two *D. auratus*, one *Am. varanensis* and one *D. compactus* identified only morphologically. The spectra of the ticks introduced in the
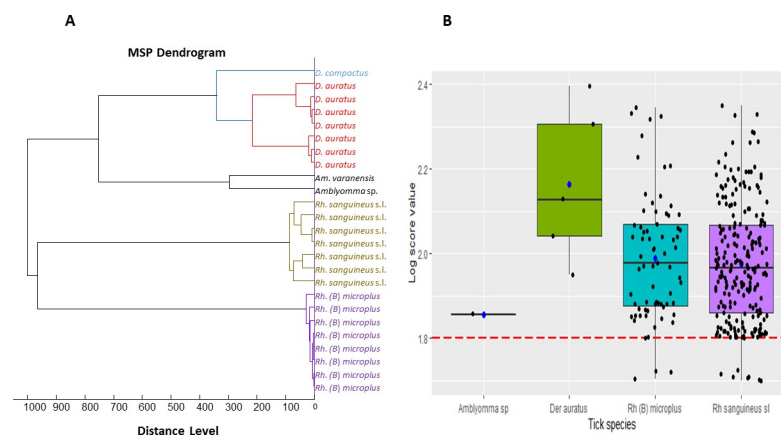
Fig 3. Comparison of MALDI-TOF MS spectra from the legs of six alcohol-preserved tick species collected in Vietnam and stored for different periods of time. The dendrogram was built using between one and eight representative MS spectra from six distinct tick species (A). The MS spectra of different specimens showed intra-species reproducibility and inter-species specificity as confirmed by PCA (B).

https://doi.org/10.1371/journal.pntd.0009813.g003

38

MALDI-TOF MS database have been deposited on the website of the University Hospital Institute (UHI) under the following DOI: https://doi.org/10.35088/rbqp-g648. The blind test revealed that 100% (233) of *Rh. sanguineus* s.l. specimens were correctly identified as *Rh. sanguineus* s.l. with LSVs ranging from 1.7–2.351 with a mean of 1.976 ± 0.137, 100% (71) of *Rh.* (*B*) *microplus* identified with LSVs ranging from 1.705–2.346 with a mean of 1.989 ± 0.148 and 100% (five) *D. auratus* with LSVs of 1.949–2.396 with a mean of 2.164 ± 0.149 (Table 2). The tick identified morphologically as *Amblyomma* sp. was identified by MALDI-TOF MS as *Am. varanensis* (LSV = 1.857) (Table 2). All our specimens were identified with LSVs ranging from 1.7–2.396 with a mean of 1.982 ± 0.142 and a median of 1.971, and 97% (301) had LSVs >1.8, which is considered the threshold for identification (Fig 3B). No blind test was performed for *D. compactus* because of the low number of specimens.

Detection of microorganisms in ticks

A total of 361 ticks, including 260 (72%) non-engorged and 101 (28%) engorged ticks, were examined for the DNA of six microorganisms using qPCR. Thirty-nine (10.8%) were positive for at least one of the microorganisms, including Anaplasmataceae, *Rickettsia* spp, *Borrelia* spp. and Piroplasmida (Table 3). Notably, two *Rh.* (*B*) *microplus* specimens were co-infected with both Anaplasmataceae and Piroplasmida. No samples were positive for *C. burnetii* or *Bartonella* spp.

DNA from bacteria of the Anaplasmataceae family were detected in 18/361 (5%) of ticks by qPCR. The DNA of bacteria belonging to the Anaplasmataceae family was found in 13 (72%) *Rh.* (*B*) *microplus* and five (28%) *Rh. sanguineus* s.l. We successfully obtained seven (40%) sequences all from *Rh.* (*B*) *microplus* by standard PCR and sequencing using the *23S* Anaplasmataceae gene amplifying a 520-pb fragment of rRNA (Table 3). A BLAST analysis showed that four of the sequences obtained were 100% identical to the corresponding sequence of *Anaplasma marginale* (Genbank: CP023731), one of sequences obtained was 100% identical to the corresponding sequence of *Ehrlichia rustica* (Genbank: KT364330), one was 99.13% identical to the corresponding sequence of *Anaplasma phagocytophilum* (Genbank: CP015376) and one was 100% identical to the corresponding sequence of *Anaplasma platys* (Genbank: CP046391).

DNA of Piroplasmida was detected in 19/361 (5.3%) of ticks by qPCR using the *5.8S* rRNA gene. Of these, ten (53%) were found in *Rh. sanguineus* s.l. and nine (47%) were found in *Rh.*

Table 3. Microorganisms detected using molecular biology tools in ticks collected in Vietnam.

Microorganisms tested	Tick species			Total
	Rh. sanguineus	*Rh. (Bo) microplus*	*Amblyomma* sp.	
Anaplasmataceae	**2% (5/251)**	**13.1% (13/99)**	-	**5% (18/361)**
Anaplasma phagocytophilum	-	1% (1/99)	-	0.3% (1/361)
Anaplasma platys	0.4% (1/251)	-	-	0.3% (1/361)
Anaplasma marginale	1.2% (3/251)	1% (1/99)	-	1.1% (4/361)
Ehrlichia rustica	-	1% (1/99)	-	0.3% (1/361)
Piroplasmida	**4% (10/251)**	**10.1% (9/99)**	-	**5.3% (19/361)**
Babesia vogeli	3.6% (9/251)	-	-	2.5% (9/361)
Theileria sinensis	-	6.1% (6/99)	-	1.7% 96/361)
Theileria orientalis	-	3% (3/99)	-	0.8% (3/361)
Rickettsia sp.	-	-	100% (1/1)	0.3% (1/361)
Borrelia sp.	0.4% (1/251)	-	-	0.3% (1/361)

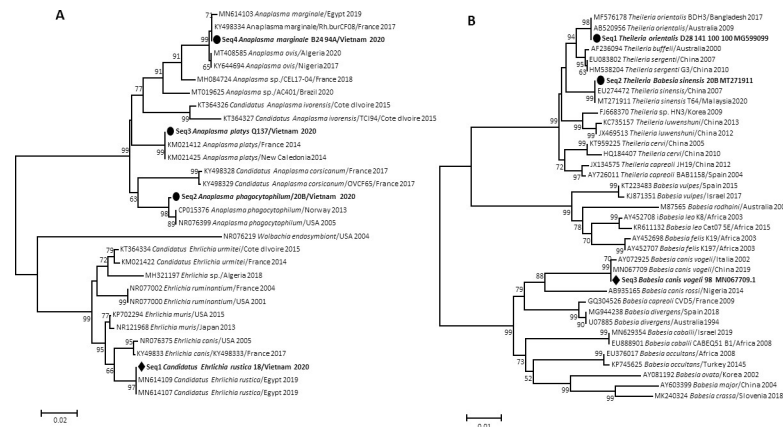https://doi.org/10.1371/journal.pntd.0009813.t003

Fig 4. *23S* rRNA gene-based phylogenetic analysis of strains identified in this study. Phylogenetic tree highlighting the position of *A. phagocytophilum*, *A. marginale*, *A. platys*, and *E. rustica* identified in our study are close to their homologues available in GenBank (A). *18S* rRNA gene-based phylogenetic analysis of strains identified in the present study. Phylogenetic tree highlighting the position of *B. vogeli*, *T. sinensis*, and *T. orientalis* relative to their correspondence available in GenBank (B).

(*B*) *microplus*. We successfully obtained 18 (95%) sequences by standard PCR and sequencing using the *18S* rRNA gene amplifying a 969-pb fragment of rRNA. The BLAST analysis of nine sequences obtained from *Rh. sanguineus* s.l. revealed that they were between 99.75% and 100% identical to the corresponding sequence of *Babesia vogeli* (GenBank: MN067709), six sequences obtained from *Rh.* (*B*) *microplus* were between 99.82% and 100% identical to the corresponding sequences of *Theileria sinensis* (GenBank: KF559355, MT271911, AB000270) and three sequences obtained from *Rh.* (*B*) *microplus* were between 99.88 and 100% identical to the corresponding sequences of *Theileria orientalis* (GenBank: MG599099) (Table 3).

Rickettsia and *Borrelia* sp. were detected by qPCR in one tick of *Amblyomma* sp. and one of *Rh. sanguineus* s.l., respectively. However, all the standard PCR procedures for the identification of *Rickettsia* and *Borrelia* species failed. Of the 25 ticks for which we obtained sequences of microorganisms, 16 (64%) came from engorged ticks and one tick (4%) was co-infected with *A. phagocytophilum* and *T. sinensis*. The species of microorganism, the species of tick and the state of engorgement of the ticks in which the microorganisms were detected are listed in S1 Table.

Two phylogenetic trees of Anaplasmataceae and Piroplasmida were built from the *23S* rRNA and *18S* rRNA genes sequences of our amplicons, respectively. These phylogenetic trees showed that the microorganisms detected in this study are close to their homologues available in GenBank (Fig 4A and 4B).

Discussion

The correct identification of tick species and associated pathogens can contribute to improving vector control efforts adapted to the surveillance and prevention of outbreaks of tick-borne diseases. In this study, our ticks were identified using traditional methods (morphological) and then confirmed by molecular methods and MALDI-TOF MS, and the associated pathogens were researched using molecular tools. In this study, we combined these three tools to identify ticks and to search for microorganisms associated with these ticks collected in Vietnam.

In this study, the morphological identification of ticks collected in Vietnam revealed six species, including *Rh. sanguineus* s.l., *Rh.* (*B*) *microplus*, *Am. varanensis*, *Amblyomma* sp., *D.*

40

auratus and *D. compactus*. All these species had already been reported in Vietnam [3, 19, 25] and neighbouring countries including Laos, Malaysia, Cambodia, and Thailand [3, 23, 43]. Among the *Rh. sanguineus* s.l. were the species most commonly found on dogs in Vietnam. This tick species is the most widely distributed worldwide and is known to be a vector of several pathogens such as *Anaplasma*, *Rickettsia*, *Ehrlichia*, and *Babesia* spp. [44, 45]. *Rhipicephalus* (*Boophilus*) *microplus* was collected from both cows and goats and is responsible for the transmission of livestock pathogens [6, 24]. There have been several reports of tick-borne livestock pathogens such as *Anaplasma* spp., *Ehrlichia ruminantium*, *Babesia bigemina*, *Babesia bovis*, and *Theileria* spp. [46–48]. However, this tick rarely bites humans [22]. Other tick species were collected from wild animals (pangolins and pigs). Several species of ticks of the genus *Amblyomma* have been collected from almost all species of pangolins [49, 50] and are vectors of *Rickettsia*, *Ehrlichia* spp. [51]. Recently, several studies reported *Amblyomma javanense* detected from pangolins in Singapore [52] and China [53], and *Amblyomma compressum* ticks on pangolins from Congo [54]. Our study is the first to observe *Am. varanensis*, *Amblyomma* sp. on pangolins from Vietnam. *Dermacentor auratus*, *D. compactus* are widely distributed across Sri Lanka, Bangladesh, India, and SEA including Vietnam [55, 56], and are well known vectors of *Rickettsia*, *Coxiella burnetii*, *Borreli*a, and *Anaplasma* spp. [57, 58].

Molecular techniques were used to confirm our morphological identification of tick species by amplifying a portion sequence of a 465-bp fragment *16S* rRNA gene. The choice of the *16S* rRNA gene was based on previous studies that reported that this gene was a reliable tool for tick identification [29, 59]. Interrogating the GenBank database with *16S* rDNA sequences from *Rh. sanguineus* s.l and *Rh.* (*B*) *microplus* showed similarity with the reference sequences available in Genbank for these species that were stored in 70% alcohol for approximately two years. Conversely, we were unable to obtain sequences for all specimens that had been preserved for more than 10 years in alcohol (i.e., *Am. varanensis*, *Amblyomma* sp., *D. auratus*, and *D. compactus*) with the *16S* and *12S* rDNA genes. This might be due to the fact that the alcohol was not completely eliminated during extraction [60] and/or to the fact that these ticks contained blood from their host, which includes several factors that can inhibit the PCR reaction, as already reported [61].

In this study, MALDI-TOF MS was used to identify ticks collected in Vietnam from domestic and wild animals. Among the spectra of tick legs that were subjected to MS analysis, the correct identification rates (LSVs >1.8) were 97%, almost identical to the identification rate reported in other studies [32, 33, 62]. Interestingly, specimens that were not able to be identified by molecular biology were identified by MALDI-TOF MS. This confirms that the tool is reliable and accurate for the identification of ticks. Despite these numerous advantages, this technique is limited by the high cost of the device, although it can be used for clinical microbiology and mycology in addition to entomology, with no additional cost. Maintenance may be another limitation but this can be compensated for by the low cost of reagents once the device is acquired [30]. Secondly, the development of protocols, the choice of the arthropod compartment to be used, the spectra for the creation of the database and, finally, the methods and time of conservation of the arthropods can influence the performance of MALDI-TOF MS [30, 37, 63].

In this study, 10.8% of the ticks were positive for at least one of the microorganisms by qPCR, of which 16/25 (64%) of the ticks carrying DNA of microorganisms by sequencing were engorged ticks. The detection of microorganisms in engorged ticks doesn't have the same epidemiological meaning as when detected in a questing or non-engorged attached tick. Such ticks may potentially have fed on hosts with bacteraemia, thus biasing the estimate of the actual rate of tick infestation.

The microorganisms detected in this study and confirmed by sequencing belong to the Anaplasmataceae family (*A. phagocytophilum*, *A. marginale*, *A. platys*, and *E. rustica*), which

41

are known aetiologies of zoonotic diseases [8, 13, 64, 65]. The Piroplasmida family (*B. vogeli*, *T. sinensis*, and *T. orientalis*) was mainly known as the potential zoonotic pathogens [66].

Anaplasma marginale is responsible for bovine anaplasmosis and is an intracellular bacterium transmitted by tick species mainly belonging to the *Rhipicephalus* and *Dermacentor* genera [67]. The DNA and specific antibodies against *A. marginale* were previously reported in the blood of cattle and cows from Vietnam [23, 24]. This study is the first report of *A. marginale* in *Rh. (B) microplus* and *Rh. sanguineus* s.l ticks collected in Vietnam. However, *A. marginale* had previously been reported in cattle and cattle *Rh. (B) microplus* ticks in China [68], the Philippines [69] which is a neighbouring country to Vietnam, in cattle and cattle ticks in Malaysia [70], and many African countries [71].

Anaplasma platys, the aetiological agent of infectious canine cyclic thrombocytopenia and which can be transmitted by *Rh. sanguineus* s.l., *A. platys* has been recorded in China [48], Colombia [72], and detected on various ectoparasites such as *Rh. (B) microplus* [48] and *Hyalomma dromedarii* [73]. *Anaplasma platys* is one of the most significant tick-borne zoonotic pathogens [24, 74] and several cases of human infections have been described in Venezuela [75], Chicago [76], and South Africa [77]. *Anaplasma platys* has already been detected from blood specimens of cattle and dogs in Vietnam [24], but it was the first discovery in *Rh. sanguineus* s.l. ticks from Vietnam in our study. It had been previously detected in *Rh. sanguineus* s.l. in SEA [25], including in the Philippines [78], Thailand, and Malaysia [79, 80].

The pathogen *A. phagocytophilum* is the causative agent of human granulocytic anaplasmosis (HGA) and tick-borne fever in ruminants [81]. It is rarely found in *Rh. (B) microplus* and is known to be transmitted by the *Ixodes* tick genus [82]. Of the detected tick-borne diseases, *A. phagocytophilum* is the most important bacterium due to its wide distribution across Europe, Asia, and North America [83, 84], with several reports of human infections [85, 86]. This is the first study reporting the detection of *A. phagocytophilum* in *Rh. (B) microplus* ticks using the molecular method in Vietnam. It has also been described in the same tick species in China [87] and Malaysia [70].

We found *Candidatus Ehrlichia rustica* in the *Ehrlichia chaffeensis* group, the agent of human monocytic ehrlichiosis [88]. Canine ehrlichiosis was first recorded in a serological study in US military dogs serving in the Vietnam war [89]. The vectors of this pathogen are *Rhipicephalus*, *Amblyomma*, *Dermacentor* spp. [90]. Another study from 2003 reported that *Ehrlichia* spp., which gathered with *E. chaffeensis*, was also discovered in other species, such as *Haemaphysalis hystricis* from wild pigs in Vietnam [22], and *Ixodes sinensis* in China [91].

Babesia vogeli, the agent of canine babesiosis in North and South America, is transmitted by *Rh. sanguineus* s.l. and is the less pathogenic species. It is a protozoan found mainly in tropical or subtropical areas of northern, eastern and southern Africa, Asia, and northern and central Australia [92]. In SEA, *B. vogeli* has been described in Malaysia [93] and in the Philippines [94]. The molecular evidence of *B. vogeli* in *Rh. sanguineus* s.l. collected from dogs has been reported in Vietnam [4] and in ticks collected from East and Southeast Asia [25]. The DNA of *B. vogeli* was detected in this study in *Rh. sanguineus* s.l. ticks, confirming the presence of the protozoan in Vietnam.

Theileria sinensis, the causative agent of bovine theileriosis, causes economic losses and threats to the cattle industry. *Theileria sinensis* is primarily distributed throughout Asia (including China, the Korean Peninsula, Japan, and Malaysia [95–97]. It was identified in *Haemaphysalis qinghaiensis* ticks collected from cattle and yaks in China [98]. *Theileria* spp. were then detected in *Haemaphysalis longicornis*, *Hyalomma* (i.e., *Hy. detritum*, *Hy. dromedarii*, *Hy. a. anatolicum*, *Hy.a asiaticum*, *Hy. rufipes*), and *Rhipicephalus* sp. [99, 100]. Besides ticks, *Theileria* spp. were also detected in sheep, goat, and ruminant blood samples [101]. This is the first report of *T. sinensis* DNA in *Rh. (B) microplus* in Vietnam.

42

Similarly, *Theileria orientalis*, the causative agent of oriental theileriosis, is an economically significant protozoan which infects cattle [95]. *Theileria orientalis* is widely distributed in countries such as Japan [102], China [103], Indonesia [104], Australia [105], and New Zealand [95]. The *Theileria orientalis* species has been identified in Vietnam from blood samples from cattle, water buffalo, sheep, goats and *Rh. (B) microplus* ticks collected from these hosts [46]. Here, we showed the presence of 3% *T. orientalis* in *Rh. (B) microplus* collected from cows. Although *Rh. (B) microplus* is not recorded as a vector of *T. orientalis*, none of the common vectors *Amblyomma*, *Dermacentor*, and *Haemaphysalis* spp. [106] were detected in our work.

Rickettsia spp. and *Borrelia* spp. detected by qPCR in this study were not amplified and sequenced to confirm their species. As previously reported, this could be caused by the higher sensitivity of qPCR than standard PCR [107].

Co-infections in ticks usually occur after a blood meal from a host co-infected with different microorganisms. In this study, we reported for the first time the co-infection by *A. phagocytophilum* and *T. sinensis* in *Rh. (B) microplus* ticks. The coinfection rate of 0.3% (1/361) in this study is lower those that have been reported in the Côte d'Ivoire [71], and in Mali [33].

Conclusion

Our work indicates that MALDI-TOF MS is a useful and reliable tool for the identification of alcohol-preserved tick species which have undergone different storage periods collected in Vietnam. Our database demonstrates, for the first time, the prevalence of *A. platys*, *A. phagocytophilum*, *A. marginale*, *E. rustica*, and *T. sinensis* pathogens in ticks collected in Vietnam. Our finding should prompt further investigation to evaluate the potential risks of ticks and tick-associated pathogens in Vietnam. Furthermore, it shows that MALDI-TOF MS may be used as an alternative tool for identifying ticks infected or uninfected by pathogens in future studies.

Supporting information

S1 Fig. Flow diagram of tick specimens which were included and analysed using MALDI-TOF MS and molecular tools.
(TIF)

S1 Table. The number of microorganisms were detecetd in engorged/non-engored ticks.
*: Tick was co-infections by two microoganisms.
(DOCX)

Author Contributions

Conceptualization: Ly Na Huynh, Nhiem Le-Viet, Philippe Parola.

Data curation: Ly Na Huynh, Adama Zan Diarra, Philippe Parola.

Formal analysis: Ly Na Huynh, Adama Zan Diarra, Philippe Parola.

43

References

1. Parola P, Raoult D. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. *Clin Infect Dis*. 2001; 32(6): 897–928. https://doi.org/10.1086/319347 PMID: 11247714

2. Otranto D, Dantas-Torres F, Breitschwerdt EB. Managing canine vector-borne diseases of zoonotic concern: part one. *Trends Parasitol*. 2009; 25(4): 157–63. https://doi.org/10.1016/j.pt.2009.01.003 PMID: 19269898

3. Petney TN, Kolonin GV, Robbins RG. Southeast Asian ticks (Acari: Ixodida): a historical perspective. *Parasitol Res*. 2007; 101: S201–05. https://doi.org/10.1007/s00436-007-0687-4 PMID: 17823829

4. Nguyen VL, Colella V, Iatta R, Bui KL, Dantas-Torres F, Otranto D. Ticks and associated pathogens from dogs in northern Vietnam. *Parasitol Res*. 2019; 118(1): 139–142. https://doi.org/10.1007/s00436-018-6138-6 PMID: 30421349

5. Dantas-Torres F. The brown dog tick, Rhipicephalus sanguineus (Latreille, 1806) (Acari: Ixodidae): from taxonomy to control. *Vet Parasitol*. 2008; 152(3–4): 173–185. https://doi.org/10.1016/j.vetpar.2007.12.030 PMID: 18280045

6. Sungirai M, Moyo DZ, Clercq PD, Madder M, Vanwambeke SO, Clercq EMD. Modelling the distribution of Rhipicephalus microplus and R. decoloratus in Zimbabwe. *Vet Parasitol Reg Stud Reports*. 2018; 14: 41–49. https://doi.org/10.1016/j.vprsr.2018.08.006 PMID: 31014735

7. Kaewmongkol G, Lukkana N, Yangtara S, Sirinarumitr T, Jittapalapong S, Fenwick SG, et al. Association of Ehrlichia canis, Hemotropic Mycoplasma spp. and Anaplasma platys and severe anemia in dogs in Thailand. *Vet Microbiol*. 2017; 201: 195–200. https://doi.org/10.1016/j.vetmic.2017.01.022 PMID: 28284610

8. Koh FX, Panchadcharam C, Sitam FT, Tay ST. Molecular investigation of Anaplasma spp. in domestic and wildlife animals in Peninsular Malaysia. *Vet Parasitol Reg Stud Reports*. 2018; 13: 141–147. https://doi.org/10.1016/j.vprsr.2018.05.006 PMID: 31014863

9. Apanaskevich MA, Apanaskevich DA. Description of New Dermacentor (Acari: Ixodidae) Species from Malaysia and Vietnam. *J Med Entomol*. 2015; 52(2): 156–62. https://doi.org/10.1093/jme/tjv001 PMID: 26336300

10. Petney T, Saijuntha W, Boulanger N, Muders SV, Petney DA, Robbins RG, et al. Ticks (Argasidae, Ixodidae) and tick-borne diseases of continental Southeast Asia. *Zootaxa*. 2019; 4558:1. https://doi.org/10.11646/zootaxa.4558.1.1 PMID: 30790915

11. Statista. Agriculture in Vietnam—Statistics & Facts.[cited 23 2021]. Available from: https://www.statista.com/topics/5653/agriculture-in-vietnam.

12. FAO. Small family farms country factsheet," 2018. [cited 2 Oct 2020]. Available from: http://www.fao.org/3/I8358EN/i8358en.pdf.

13. Dantas-Torres F, Chomel BB, Otranto D. Ticks and tick-borne diseases: a One Health perspective. *Trends Parasitol*. 2012; 28(10): 437–46. https://doi.org/10.1016/j.pt.2012.07.003 PMID: 22902521

44

14. Kwaghe A, Teru V, Ndahi M, Usman J, Abubakar A, Iwar V. Veterinary Services as a Panacea for Agricultural Development and Increase in Nigeria's Gross Domestic Product (GDP): A Review. *International Journal of Life Sciences*. 2015; 4(2): 134–46.

15. Chin S. Thriving dog meat trade | The ASEAN Post. [cited 21 Mar 2021]. Available from: https://theaseanpost.com/article/thriving-dog-meat-trade.

16. Ngo TC, Nguyen DT, Tran HH, Morita K, Nguyen TH, Ehara M, et al. Imported Dogs as Possible Vehicles of Vibrio Cholerae O1 Causing Cholera Outbreaks in Northern Vietnam. *The Open Infectious Diseases Journal*. 2011; 5: 127–34. https://doi.org/10.2174/1874279301105010127

17. Gebrekidan H, Nelson LG. Smith RB. Gasser, Jabbar A. An outbreak of oriental theileriosis in dairy cattle imported to Vietnam from Australia. *Parasitology*. 2017; 144(6): 738–46. https://doi.org/10.1017/S0031182016002328 PMID: 27938442

18. Sivakumar T, Dinh TBL, Phung TL, Keisuke S, Ikuo I, Naoaki Y, et al. Serological and molecular surveys of Babesia bovis and Babesia bigemina among native cattle and cattle imported from Thailand in Hue, Vietnam. *J Vet Med Sci*. 2018; 80(2): 333–6. https://doi.org/10.1292/jvms.17-0549 PMID: 29249730

19. Kolonin GV. Review of the Ixodid tick fauna (Acari: Ixodidae) of Vietnam. *J Med Entomol*. 1995; 32(3): 276–82. https://doi.org/10.1093/jmedent/32.3.276 PMID: 7616517

20. Apanaskevich DA, Apanaskevich MA. Description of a New Dermacentor (Acari: Ixodidae) Species from Thailand and Vietnam. *J Med Entomol*. 2015b; 52(5): 806–12. https://doi.org/10.1093/jme/tjv067 PMID: 26336207

21. Petney TN, Keirans JE. Ticks of the genera Boophilus, Dermacentor, Nosomma and Rhipicephalus (Acari: Ixodidae) in South-east Asia. *Tropical Biomedicine*. 1996a; 13: 73–84. [cited 30 Mar 2021]. Available from: https://www.scienceopen.com/document?vid=e77f85d6-9946-4217-87e4-0dc7e1c850dd.

22. Parola P, Cornet JP, Sanogo YO, Raoult D, Telford SR III, Wongsrichanalai C, et al. Detection of Ehrlichia spp., Anaplasma spp., Rickettsia spp., and other eubacteria in ticks from the Thai-Myanmar border and Vietnam. *J Clin Microbiol*. 2003; 4(4): 1600–08. https://doi.org/10.1128/jcm.41.4.1600–1608.2003

23. Geurden T, Somers R, Thanh NTG, Dorny P, Giao HK, Vercruysse J, et al. Parasitic infections in dairy cattle around Hanoi, northern Vietnam. *Vet Parasitol*. 2008; 153(3–4): 384–88. https://doi.org/10.1016/j.vetpar.2008.01.031 PMID: 18328629

24. Chien NTH, Nguyen TL, Bui KL, Nguyen VT, Le TH. Anaplasma marginale and A. platys Characterized from Dairy and Indigenous Cattle and Dogs in Northern Vietnam. *Korean J Parasitol*. 2019; 57 (1): 43–47. https://doi.org/10.3347/kjp.2019.57.1.43 PMID: 30840799

25. Nguyen VL, Colella V, Greco G, Hadi UK, Venturina V, Tong KBY, et al. Molecular detection of pathogens in ticks and fleas collected from companion dogs and cats in East and Southeast Asia. *Parasit Vectors*. 2020; 13(1): 420. https://doi.org/10.1186/s13071-020-04288-8 PMID: 32799914

26. Mediannikov O and Fenollar F. Looking in ticks for human bacterial pathogens. *Microb Pathog*. 2014; 77: 142–8. https://doi.org/10.1016/j.micpath.2014.09.008 PMID: 25229617

27. Lauri A, Mariani PO. Potentials and limitations of molecular diagnostic methods in food safety. *Genes Nutr*. 2009; 4(1): 1–12. https://doi.org/10.1007/s12263-008-0106-1 PMID: 19067016

28. Favrot C. Polymerase chain reaction: advantages and drawbacks. *The Zurich Open Repository and Archive*, *University of Zurich*. 2015. [cited 18 Dec 2015]. Available from: https://doi.org/https%3A//doi.org/10.5167/uzh-116536

29. Yssouf A, Almeras L, Raoult D, Parola P. Emerging tools for identification of arthropod vectors. *Future Microbiol*. 2016; 11(4): 549–66. https://doi.org/10.2217/fmb.16.5 PMID: 27070074

30. Sevestre J, Diarra AZ, Laroche M, Almeras L, Parola P. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: an emerging tool for studying the vectors of human infectious diseases. *Future Microbiol*. 2021; 16: 323–40. https://doi.org/10.2217/fmb-2020-0145 PMID: 33733821

31. Berry CM. Resolution of the taxonomic status of Rhipicephalus (Boophilus) microplus. Institute of Biodiversity, Animal Health and Comparative Medicine College of Medical, Veterinary and Life Sciences University of Glasgow. 2017; 268.

32. Boyer PH, Almeras L, Plantard O, McCoy K, Jaulhac B, Boulanger N, et al. Identification of closely related Ixodes species by protein profiling with MALDI-TOF mass spectrometry. *PLoS One*. 2019; 14 (10): e0223735. https://doi.org/10.1371/journal.pone.0223735 PMID: 31622384

33. Diarra AZ, Almeras L, Laroche M, Doumbo O, Raoult D, Parola P, et al. Molecular and MALDI-TOF identification of ticks and tick-associated bacteria in Mali. *PLoS Negl Trop Dis*. 2017; 11(7). e0005762. https://doi.org/10.1371/journal.pntd.0005762 PMID: 28742123

34. Kumsa B, Laroche M, Almeras L, Mediannikov O, Raoult D, Parola P. Morphological, molecular and MALDI-TOF mass spectrometry identification of ixodid tick species collected in Oromia, Ethiopia. *Parasitol. Res*. 2016; 115(11): 4199–210. https://doi.org/10.1007/s00436-016-5197-9 PMID: 27469536

35. Boucheikhchoukh M, Laroche M, Aouadi A, Benakhla A, Raoult D, Parola P, et al. MALDI-TOF MS identification of ticks of domestic and wild animals in Algeria and molecular detection of associated microorganisms. *Comp Immunol Microbiol Infect Dis*. 2018; 57: 39–49. https://doi.org/10.1016/j.cimid.2018.05.002 PMID: 30017077

36. Yssouf A, Socolovschi C, Flaudrops C, Sokhna CS, Raoult D, Parola P, et al. Matrix-Assisted Laser Desorption Ionization—Time of Flight Mass Spectrometry: An Emerging Tool for the Rapid Identification of Mosquito Vectors. *PLoS One*. 2013; 8(8). https://doi.org/10.1371/journal.pone.0072380 PMID: 23977292

37. Nebbak A, Willcox AC, Bitam I, Raoult D, Parola P, Almeras L Standardization of sample homogenization for mosquito identification using an innovative proteomic tool based on protein profiling. *Proteomics*. 2016; 16(24): 3148–60. https://doi.org/10.1002/pmic.201600287 PMID: 27862981

38. Yssouf A, Parola P, Lindström A, Berenger JM, Raoult D, Almeras L, et al. Identification of European mosquito species by MALDI-TOF MS. *Parasitol. Res*. 2014; 113(6): 2375–78. https://doi.org/10.1007/s00436-014-3876-y PMID: 24737398

39. Dahmana H, Amanzougaghene N, Davoust B, Chik M, Fenollar F, Mediannikov O, et al. Great diversity of Piroplasmida in Equidae in Africa and Europe, including potential new species. *Vet Parasitol Reg Stud Reports*. 2019; 18: 100332. https://doi.org/10.1016/j.vprsr.2019.100332 PMID: 31796173

40. Lafri I, Hamzaoui BE, Bitam I, Karakellah M, Raoult D, Parola P, et al. Detection of relapsing fever *Borrelia* spp., *Bartonella* spp. and Anaplasmataceae bacteria in argasid ticks in Algeria. *PLoS Negl Trop Dis*. 2017; 11(11): e0006064. https://doi.org/10.1371/journal.pntd.0006064 PMID: 29145396

41. Dahmani M, Loudahi A, Mediannikov O, Fenollar F, Raoult D, Davoust B. Molecular detection of Anaplasma platys and Ehrlichia canis in dogs from Kabylie, Algeria. *Ticks Tick Borne Dis*. 2015; 6(2): 198–203. https://doi.org/10.1016/j.ttbdis.2014.12.007 PMID: 25583345

42. Vial L, Diatta G, Tall A, Rogier C, Renaud F, Trape JF, et al. Incidence of tick-borne relapsing fever in west Africa: longitudinal study. *Lancet*. 2006; 368(9529): 37–43. https://doi.org/10.1016/S0140-6736(06)68968-X PMID: 16815378

43. Vongphayloth K, Hertz JC, Lakeomany K, Robbins RG, Sutherland IW, Brey PT, et al. The Genus Dermacentor (Acari: Ixodidae) in Laos: A Review and Update of Species Records. *J Med Entomol*. 2018; 55(4): 1047–50. https://doi.org/10.1093/jme/tjy041 PMID: 29590396

44. Parola P, Paddock CD, Raoult D. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. *Clin Microbiol Rev*. 2005; 18(4): 719–56. https://doi.org/10.1128/CMR.18.4.719-756.2005 PMID: 16223955

45. Dantas-Torres F, Otranto D. Further thoughts on the taxonomy and vector role of Rhipicephalus sanguineus group ticks. *Vet Parasitol*. 2015; 208(1–2): 9–13. https://doi.org/10.1016/j.vetpar.2014.12.014 PMID: 25579394

46. Khukhuu A, Dinh TBL, Phung TL, Igarashi I, Xuan X, Yokoyama N, et al. Molecular epidemiological survey of Theileria orientalis in Thua Thien Hue Province, Vietnam. *J Vet Med Sci*. 2011; 73(5): 701–5. https://doi.org/10.1292/jvms.10-0472 PMID: 21187678

47. Adjou Moumouni PF, Aplogan GL, Katahira H,Wang G, Mingming Liu M, Xuan X, et al. Prevalence, risk factors, and genetic diversity of veterinary important tick-borne pathogens in cattle from Rhipicephalus microplus-invaded and non-invaded areas of Benin. *Ticks Tick Borne Dis*. 2018; 9(3): 450–64. https://doi.org/10.1016/j.ttbdis.2017.12.015 PMID: 29307783

48. Guo WP, Zhang B, Wang YH, Xu G, Wang X, Ni X, et al. Molecular identification and characterization of Anaplasma capra and Anaplasma platys-like in Rhipicephalus microplus in Ankang, Northwest China. *BMC Infect Dis*. 2019; 19(1): 434. https://doi.org/10.1186/s12879-019-4075-3 PMID: 31101084

49. Hassan M, Sulaiman MH, Lian CJ. The prevalence and intensity of Amblyomma javanense infestation on Malayan pangolins (Manis javanica Desmarest) from Peninsular Malaysia. *Acta Trop*. 2013; 126 (2): 142–5. https://doi.org/10.1016/j.actatropica.2013.02.001 PMID: 23416121

50. Khatri-Chhetri R, Wang HC, Chen CC, Khatri-Chhetri N, Wu HY, Pei KJC, et al. Surveillance of ticks and associated pathogens in free-ranging Formosan pangolins (Manis pentadactyla pentadactyla). *Ticks Tick Borne Dis*. 2016; 7(6): 1238–44. https://doi.org/10.1016/j.ttbdis.2016.07.007 PMID: 27426438

51. Qiu Y, Kidera N, Hayashi M, Fujishima K, Tamura H. Rickettsia spp. and Ehrlichia spp. in Amblyomma ticks parasitizing wild amphibious sea kraits and yellow-margined box turtles in Okinawa, Japan. *Ticks Tick Borne Dis*. 2021; 12(2): 101636. https://doi.org/10.1016/j.ttbdis.2020.101636 PMID: 33360921

46

52. Kwak ML, Hsu CD, Douay G, Ahmad AA. The first authenticated record of the pangolin tick Amblyomma javanense (Acari: Ixodidae) in Singapore, with notes on its biology and conservation. *Exp Appl Acarol*. 2018; 76(4): 551–7. https://doi.org/10.1007/s10493-018-0310-7 PMID: 30298232

53. Jabin G, Dewan Y, Khatri H, Singh SK, Chandra K, Thakur M. Identifying the tick Amblyomma javanense (Acari: Ixodidae) from Chinese pangolin: generating species barcode, phylogenetic status and its implication in wildlife forensics. *Exp Appl Acarol*. 2019; 78(3): 461–7. https://doi.org/10.1007/s10493-019-00393-1 PMID: 31168752

54. Mediannikov O, Davoust B, Socolovschi C, Tshilolo L, Raoult D, Parola P. Spotted fever group rickettsiae in ticks and fleas from the Democratic Republic of the Congo. *Ticks Tick Borne Dis*. 2012; 3(5–6): 371–3. https://doi.org/10.1016/j.ttbdis.2012.10.015 PMID: 23137572

55. Hoogstraal G, Wassef HY. Dermacentor (Indocentor) auratus (Acari: Ixodoidea: Ixodidae): hosts, distribution, and medical importance in tropical Asia. *J Med Entomol*. 1985; 22(2): 170–7. https://doi.org/10.1093/jmedent/22.2.170 PMID: 3838555

56. Chen Z, Yang X, Bu F, Yang X, Yang X, Liu J. Ticks (acari: ixodoidea: argasidae, ixodidae) of China. *Exp Appl Acarol*. 2010; 51(4): 393–404. https://doi.org/10.1007/s10493-010-9335-2 PMID: 20101443

57. Sumrandee C, Baimai V, Trinachartvanit W, Ahantarig A. Molecular detection of Rickettsia, Anaplasma, Coxiella and Francisella bacteria in ticks collected from Artiodactyla in Thailand. *Ticks Tick Borne Dis*. 2016; 7(5): 678–89. https://doi.org/10.1016/j.ttbdis.2016.02.015 PMID: 26934997

58. Nooroong P, Trinachartvanit W, Baimai V, Ahantarig A. Phylogenetic studies of bacteria (Rickettsia, Coxiella, and Anaplasma) in Amblyomma and Dermacentor ticks in Thailand and their co-infection. *Ticks Tick Borne Dis*. 2018; 9(4): 963–71. https://doi.org/10.1016/j.ttbdis.2018.03.027 PMID: 29610046

59. Dantas-Torres F, Latrofa MS, Annoscia G, Giannelli A, Parisi A, Otranto D. Morphological and genetic diversity of Rhipicephalus sanguineus sensu lato from the New and Old Worlds. *Parasit Vectors*. 2013; 6: 213. https://doi.org/10.1186/1756-3305-6-213 PMID: 23880226

60. Schrader C, Schielke A, Ellerbroek L, Johne R. PCR inhibitors—occurrence, properties and removal. *J Appl Microbiol*. 2012; 113(5): 1014–26. https://doi.org/10.1111/j.1365-2672.2012.05384.x PMID: 22747964

61. Rodríguez González I, Fraga J, Noda AA, Duarte Y, Echevarria E, Fernández C, et al. An Alternative and Rapid Method for the Extraction of Nucleic Acids from Ixodid Ticks by Potassium Acetate Procedure. *Braz. Arch. Biol. Technol*. 2014; 57: 542–7. https://doi.org/10.1590/S1982-88372014000100011

62. Tran NHB, Nguyen HH. Tinh hinh nhiem ngoai ky sinh trung tren cho tai thanh pho Can Tho. *Can Tho Junior of Science*. 2014; 2: 69–73.

63. Nebbak A, Hamzaoui BE, Berenger JM, Raoult D, Almeras L, Parola P, et al. Comparative analysis of storage conditions and homogenization methods for tick and flea species for identification by MALDI-TOF MS. *Med Vet Entomol*. 2017; 31(4): 438–48. https://doi.org/10.1111/mve.12250 PMID: 28722283

64. Dumler JS, Barbet AF, Bekker CP, Ray SC, Rikihisa Y, Rurangirwa FR, et al. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of Ehrlichia with Anaplasma, Cowdria with Ehrlichia and Ehrlichia with Neorickettsia, descriptions of six new species combinations and designation of Ehrlichia equi and 'HGE agent' as subjective synonyms of Ehrlichia phagocytophila. *Int J Syst Evol Microbiol*. 2001; 51(Pt 6): 2145–65. https://doi.org/10.1099/00207713-51-6-2145 PMID: 11760958

65. Heppner DG, Wongsrichanalai C, Walsh DS, Eamsila C, Hanson B, Paxton H, et al. Human ehrlichiosis in Thailand. *Lancet*. 1997; 350(9080): 785–6. https://doi.org/10.1016/S0140-6736(05)62571-8 PMID: 9298007

66. Schnittger L, Rodriguez AE, Florin-Christensen M, Morrison DA. Babesia: a world emerging. *Infect Genet Evol*. 2012; 12(8): 1788–809. https://doi.org/10.1016/j.meegid.2012.07.004 PMID: 22871652

67. Battilani M, Balboni DAS A, Dondi F. Genetic diversity and molecular epidemiology of Anaplasma. *Infect Genet Evol*. 2017; 49: 195–211. https://doi.org/10.1016/j.meegid.2017.01.021 PMID: 28122249

68. Wen B, Jian R, Zhang Y, Chen R. Simultaneous detection of Anaplasma marginale and a new Ehrlichia species closely related to Ehrlichia chaffeensis by sequence analyses of 16S ribosomal DNA in Boophilus microplus ticks from Tibet. *J Clin Microbiol*. 2002; 40(9): 3286–90. https://doi.org/10.1128/JCM.40.9.3286-3290.2002 PMID: 12202567

69. Ybañez AP, Sivakumar T, Ybañez RHD, Matsumoto K, Yokoyama N, Inokuma H, et al. First molecular characterization of Anaplasma marginale in cattle and Rhipicephalus (Boophilus) microplus ticks in Cebu, Philippines. *J Vet Med Sci*. 2013; 75(1): 27–36. https://doi.org/10.1292/jvms.12-0268 PMID: 22878542

47

70. Tay ST, Koh FX, Kho KL, Ong BL. Molecular survey and sequence analysis of Anaplasma spp. in cattle and ticks in a Malaysian farm. *Trop Biomed*. 2014; 31(4): 769–76. PMID: 25776603

71. Ehounoud CB, Yao KP, Dahmani M, Raoult D, Fenollar F, Mediannikov O, et al. Multiple Pathogens Including Potential New Species in Tick Vectors in Côte d'Ivoire. *PLoS Negl Trop Dis*. 2016; 10(1): e0004367. https://doi.org/10.1371/journal.pntd.0004367 PMID: 26771308

72. Pesapane R, Foley J, Thomas R, Castro LR. Molecular detection and characterization of Anaplasma platys and Ehrlichia canis in dogs from northern Colombia. *Vet Microbiol*. 2019; 233: 184–9. https://doi.org/10.1016/j.vetmic.2019.05.002 PMID: 31176406

73. Selmi R, Ben Said M, Dhibi M, Ben Yahia H, Messadi L. Improving specific detection and updating phylogenetic data related to Anaplasma platys-like strains infecting camels (Camelus dromedarius) and their ticks. *Ticks Tick Borne Dis*. 2019; 10(6): 101260. https://doi.org/10.1016/j.ttbdis.2019.07.004 PMID: 31327747

74. Wei W, Li J, Wang YW, Cui XM, Li LF, Yuan TT, et al. Anaplasma platys-Like Infection in Goats, Beijing, China. *Vector Borne Zoonotic Dis*. 2020; 20(10): 755–62. https://doi.org/10.1089/vbz.2019.2597 PMID: 32679008

75. Arraga-Alvarado CM, Qurollo BA, Parra OC, Berrueta MA, Hegarty BC, and Breitschwerdt EB. Molecular Evidence of Anaplasma platys Infection in Two Women from Venezuela. *The American Journal of Tropical Medicine and Hygiene*. 2014; 91(6): 1161–65. https://doi.org/10.4269/ajtmh.14-0372 PMID: 25266347

76. Breitschwerdt EB, Hegarty BC, Qurollo BA, Maggi RG, Blanton LS, Bouyer DH, et al. Intravascular persistence of Anaplasma platys, Ehrlichia chaffeensis, and Ehrlichia ewingii DNA in the blood of a dog and two family members. *Parasites Vectors*. 2014; 7(1): 298. https://doi.org/10.1186/1756-3305-7-298 PMID: 24984562

77. Maggi RG, Mascarelli PE, Havenga LN, Naidoo V, Breitschwerdt EB. Co-infection with Anaplasma platys, Bartonella henselae and Candidatus Mycoplasma haematoparvum in a veterinarian. *Parasit Vectors*. 2013; 6: 103. https://doi.org/10.1186/1756-3305-6-103 PMID: 23587235

78. Ybanez A. First report on Anaplasma platys infection in a dog in the Philippines. *Israel Journal of Veterinary Medicine*. 2013; 7: 227–231.

79. Low VL, Prakash BK, Lim YAL, Vinnie-Siow WY, Sofian-Azirun M, AbuBakar S, et al. Detection of Anaplasmataceae agents and co-infection with other tick-borne protozoa in dogs and Rhipicephalus sanguineus sensu lato ticks. *Exp Appl Acarol*. 2018; 75(4): 429–35. https://doi.org/10.1007/s10493-018-0280-9 PMID: 30073430

80. Piratae S, Senawong P, Chalermchat P, Harnarsa W, Sae-chue B. Molecular evidence of Ehrlichia canis and Anaplasma platys and the association of infections with hematological responses in naturally infected dogs in Kalasin, Thailand. *Vet World*. 2019; 12(1): 131–5. https://doi.org/10.14202/vetworld.2019.131-135 PMID: 30936666

81. Stuen S, Granquist EG, Silaghi C. Anaplasma phagocytophilum—a widespread multi-host pathogen with highly adaptive strategies. *Front Cell Infect Microbiol*. 2013; 3(31). https://doi.org/10.3389/fcimb.2013.00031 PMID: 23885337

82. Ekner A, Dudek K, Sajkowska Z, Majláthová V, Majláth I, Tryjanowski P. Anaplasmataceae and Borrelia burgdorferi sensu lato in the sand lizard Lacerta agilis and co-infection of these bacteria in hosted Ixodes ricinus ticks. *Parasit Vectors*. 2011; 4: 182. https://doi.org/10.1186/1756-3305-4-182 PMID: 21933412

83. Silaghi C, Santos AS, Gomes J, Oteo JA, Fuente JDL, Dumler JS, et al. Guidelines for the Direct Detection of Anaplasma spp. in Diagnosis and Epidemiological Studies. *Vector Borne Zoonotic Dis*. 2017; 17(1): 12–22. https://doi.org/10.1089/vbz.2016.1960 PMID: 28055579

84. Mukhacheva TA, Shaikhova DR, Kovalev SY. Asian isolates of Anaplasma phagocytophilum: Multilocus sequence typing. *Ticks Tick Borne Dis*. 2019; 10(4): 775–80. https://doi.org/10.1016/j.ttbdis.2019.03.011 PMID: 30904539

85. Dumler JS, Choi KS, Garcia-Garcia JC, Garyu JW, Grab DJ, Bakken JS, et al. Human Granulocytic Anaplasmosis and Anaplasma phagocytophilum. *Emerg Infect Dis*. 2005; 11(12): 1828–34. https://doi.org/10.3201/eid1112.050898 PMID: 16485466

86. Jin H, Wei F, Liu Q, Qian J. Epidemiology and control of human granulocytic anaplasmosis: a systematic review. *Vector Borne Zoonotic Dis*. 2012; 12(4): 269–74. https://doi.org/10.1089/vbz.2011.0753 PMID: 22217177

87. Zhang L, Liu H, Xu B, Fan D, Li G, Jin Y, et al. Anaplasma phagocytophilum infection in domestic animals in ten provinces/cities of China. *Am J Trop Med Hyg*. 2012; 87(1): 185–9. https://doi.org/10.4269/ajtmh.2012.12-0005 PMID: 22764312

48

88. Rar V, Golovljova I. Anaplasma, Ehrlichia, and 'Candidatus Neoehrlichia' bacteria: pathogenicity, bio-diversity, and molecular genetic characteristics, a review. *Infect Genet Evol*. 2011; 11(8): 1842–61. https://doi.org/10.1016/j.meegid.2011.09.019 PMID: 21983560

89. Kelch WJ. The canine ehrlichiosis (tropical canine pancytopenia) epizootic in Vietnam and its implications for the veterinary care of military working dogs. *Mil Med*. 1984; 149(6): 327–31. PMID: 6429572

90. Fuente J, Estrada-Pena A, Venzal JM, Kocan KM, Sonenshine DE. Overview: Ticks as vectors of pathogens that cause disease in humans and animals. *Front Biosci*. 2008; 13: 6938–46, May 2008, https://doi.org/10.2741/3200 PMID: 18508706

91. Sun J, Liu Q, Lu L, Li G, Liu J, Lin H, et al. Coinfection with four genera of bacteria (Borrelia, Bartonella, Anaplasma, and Ehrlichia) in Haemaphysalis longicornis and Ixodes sinensis ticks from China. *Vector Borne Zoonotic Dis*. 2008; 8(6): 791–5. https://doi.org/10.1089/vbz.2008.0005 PMID: 18637722

92. Beugnet F, Moreau Y. Babesiosis. *Rev Sci Tech*. 2015; 34(2): 627–39. https://doi.org/10.20506/rst.34.2.2385 PMID: 26601462

93. Prakash BK, Low VL, Vinnie-Siow WY, Morvarid AR, AbuBakar S, Sofian-Azirun M, et al. Detection of Babesia spp. in Dogs and Their Ticks From Peninsular Malaysia: Emphasis on Babesia gibsoni and Babesia vogeli Infections in Rhipicephalus sanguineus sensu lato (Acari: Ixodidae). *J Med Entomol*. 2018; 55(5): 1337–40. https://doi.org/10.1093/jme/tjy072 PMID: 29762747

94. Ybañez AP, Ybañez RHD, Talle MG, Liu M, Moumouni PFA, Xuan X. First report on Babesia vogeli infection in dogs in the Philippines. *Parasitol Int*. 2017; 66(1): 813–5. https://doi.org/10.1016/j.parint.2016.10.001 PMID: 27713098

95. Watts JG, Playford MC, Hickey KL. Theileria orientalis: A review. *New Zealand veterinary journal*. 2015; 64(1): 1–21. https://doi.org/10.1080/00480169.2016.1090898 [cited 18 Apr 2021]. Available from: https://www.researchgate.net/publication/279864314. PMID: 26440501

96. Fujisaki K, Ito Y, Kamio T, Kitaoka S. The presence of Theileria sergenti in Haemaphysalis longicornis overwintering in pasture in Japan. *Ann Trop Med Parasitol*. 1985; 79(5): 519–24. https://doi.org/10.1080/00034983.1985.11811957 PMID: 3936424

97. Kho KL, Amarajothi ADG, Koh FX, Panchadcharam C, Hassan Nizam QN, Tay ST, "The first molecular survey of theileriosis in Malaysian cattle, sheep and goats. *Vet Parasitol Reg Stud Reports*. 2017; 10: 149–53. https://doi.org/10.1016/j.vprsr.2017.08.003 PMID: 31014589

98. Bai Q, Liu G, Yin H, Liu D, Ren J, Li X, et al. Theileria sinensis sp nov: a new species of Bovine Theileria-classical taxonomic studies. *Acta Veterinaria et Zootechnica Sinica*. 2002a; 33(2): 73–7.

99. Tian Z, Du J, Yang J, Liu X, Liu G, Yin H, et al. A PCR-RFLP Assay targeting RPS8 gene for the discrimination between bovine Babesia and Theileria species in China. *Parasit Vectors*. 2015; 8. https://doi.org/10.1186/s13071-015-1085-x PMID: 26382041

100. Li Y, Li X, Liu J, Liu G, Luo J, Yin H, el at. First Report of Theileria Infection of Bactrian Camels (Camelus bactrianus) in Xinjiang, China. *Acta Parasitol*. 2019; 64(4): 923–6. https://doi.org/10.2478/s11686-019-00086-0 PMID: 31165983

101. Yin H, Luo J, Guan G, Lu C, Yuan Z, Guo S, et al. Transmission of an unidentified Theileria species to small ruminants by Haemaphysalis qinghaiensis ticks collected in the field. *Parasitol Res*. 2002; 88 (13): S25–27. https://doi.org/10.1007/s00436-001-0565-4 PMID: 12051602

102. Kim JY, Naoaki Y, Sanjay K, Sachiko S, Kozo F, Chihiro S, et al. Molecular epidemiological survey of benign Theileria parasites of cattle in Japan: detection of a new type of major piroplasm surface protein gene. *J Vet Med Sci*. 2004; 66(3):251–6. https://doi.org/10.1292/jvms.66.251 PMID: 15107552

103. Liu A, Guan G, Liu Z, Gao J, Ma M, Niu Q, et al. Detecting and differentiating Theileria sergenti and Theileria sinensis in cattle and yaks by PCR based on major piroplasm surface protein (MPSP). *Exp Parasitol*. 2010; 126(4): 476–81. https://doi.org/10.1016/j.exppara.2010.05.024 PMID: 20685208

104. Govaerts M, Verhaert P, Jongejan F, Goddeeris BM. Characterisation of the 33kDa piroplasm surface antigen of Theileria orientalis/sergenti/buffeli isolates from West Java, Indonesia. *Vet Parasitol*. 2002; 104(2): 103–17. https://doi.org/10.1016/s0304-4017(01)00621-5 PMID: 11809330

105. Izzo MM, Poe I, Horadagoda N, De Vos AJ, and House JK. Haemolytic anaemia in cattle in NSW associated with Theileria infections. *Aust Vet J*. 2010; 88(1–2): 45–51. https://doi.org/10.1111/j.1751-0813.2009.00540.x PMID: 20148827

106. Sugimoto C, Fujisaki K. Non-Transforming Theileria Parasites of Ruminants. Springer US. 2002; 3: 93–106.

107. Kidd L, Maggi R, Diniz PPVP, Hegarty B, Tucker M, Breitschwerdt E. Evaluation of conventional and real-time PCR assays for detection and differentiation of Spotted Fever Group Rickettsia in dog blood. *Vet Microbiol*. 2008; 129(3–4): 294–303. https://doi.org/10.1016/j.vetmic.2007.11.035 PMID: 18226476

49

108. Djiba ML Mediannikov O, Mbengue M, Fenollar F, Raoult D, Ndiaye M, et al. Survey of Anaplasmata-ceae bacteria in sheep from Senegal. *Trop Anim Health Prod.* 2013; 45(7): 1557–61. https://doi.org/10.1007/s11250-013-0399-y PMID: 23553260

109. Rolain JM, Stuhl L, Maurin M, Raoult D. Evaluation of antibiotic susceptibilities of three rickettsial spe-cies including Rickettsia felis by a quantitative PCR DNA assay. *Antimicrob Agents Chemother.* 2002; 46(9): 2747–51. https://doi.org/10.1128/AAC.46.9.2747-2751.2002 PMID: 12183224

110. Mura Aet al. Molecular detection of spotted fever group rickettsiae in ticks from Ethiopia and Chad. *Trans R Soc Trop Med Hyg.* 2008; 102(9): 945–9. https://doi.org/10.1016/j.trstmh.2008.03.015 PMID: 18440576

111. Mediannikov O, Trape JF, Diatta G, Parola P, Fournier PE, Raoult D. Rickettsia africae, Western Africa. *Emerg Infect Dis.* 2010; 16(3): 571–3. https://doi.org/10.3201/eid1603.090346 PMID: 20202453

112. Rolain JM, Franc M, Davoust B, Raoult D. Molecular Detection of Bartonella quintana, B. koehlerae, B. henselae, B. clarridgeiae, Rickettsia felis, and Wolbachia pipientis in Cat Fleas, France. *Emerg Infect Dis.* 2003; 9(3): 339–42. https://doi.org/10.3201/eid0903.020278 PMID: 12643829

113. Rolain JM, Raoult D. Molecular detection of Coxiella burnetii in blood and sera during Q fever. *QJM.* 2005; 98(8): 615–7. https://doi.org/10.1093/qjmed/hci099 PMID: 16027172

50

ARTICLE III

Morphological, molecular and MALDI-TOF MS identification of flea species and their associated microorganisms in Vietnam

Ly Na Huynh, Adama Zan Diarra, Quang Luan Pham, Jean-Michel Berenger, Van Hoang Ho, Xuan Quang Nguyen, Philippe Parola*

Abstract

Fleas are obligatory blood-sucking ectoparasites of medical and veterinary importance. The identification of fleas and associated flea-borne microorganisms is thus critical in controlling and managing these vectors. However, the absence of standard taxonomic keys and reference data specific to Vietnamese flea species makes morphological identification of fleas more difficult, if not impossible. Arthropods and the associated microorganisms have been identified using molecular methods. Molecular approaches have been used to identify arthropods and their associated microorganisms. However, these techniques have a number of limitations, including the need for primer-specific targeting, the expense of doing so, and the need for GenBank reference sequence availability. Recently, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has been reported as an innovative and effective approach to the identification of arthropods, including fleas. This research aims to identify ethanol-preserved fleas collected in Vietnam by the MALDI-TOF MS tool, as well as use molecular biology to look for microorganisms associated with these fleas. A total of 502 fleas were collected mainly from cats, dogs, and small wild rodents in four provinces of Vietnam. Morphological identification led to the recognition of five flea species, namely *Xenopsylla cheopis*, *Xenopsylla astia*, *Pulex irritans*, *Ctenocephalides canis*, and *Ctenocephalides felis*. These ectoparasites are well known to be vectors of human and veterinary pathogens, which are considered important for public health worldwide. The cephalothoraxes of 300 individual, randomly selected fleas were tested by MALDI-TOF MS and molecular analysis for identification and microorganism detection. A total of 257/300 (85,7%) of the obtained spectra from cephalothoraxes of each species were of good enough quality to be used for our analyses. Our laboratory MALDI-TOF MS reference database was upgraded with spectra achieved from five randomly selected fleas for every species of

52

Ctenocephalides canis and *Ctenocephalides felis*. The remaining spectra were then queried against the upgraded MALDI-TOF MS database, which showed 100% correspondence between morphology and MALDI-TOF MS identification for two flea species (*Ctenocephalides canis* and *Ctenocephalides felis*). The MS spectra of the remaining species (three *P. irritans*, five *X. astia*, and two *X. cheopis*) were visually generated low-intensity MS profiles with high background noise that could not use to update our database. The limited sample size of these species (between two and five specimens per species) may explain our spectra results.

We report, for the first time, the presence of *Bartonella* and *Wolbachia* spp. in three flea species from Vietnam, which were detected in 300 fleas by using PCR and sequencing with primers derived from the *gltA* gene for *Bartonella* and *16S* rRNA gene for *Wolbachia*, including three *Bartonella clarridgeiae* (1%), three *Bartonella rochalimae* (1%), one *Bartonella coopersplainsensis* (0.3%), and 174 *Wolbachia* spp. Endosymbiont (58%). The detection of these pathogenic bacteria in their ectoparasites may be applied to epidemiologic surveillance and prevention strategies.

Map of Vietnam

Morphological identification

Xenopsylla cheopis

Ctenocephalides canis

Xenopsylla astia

Ctenocephalides felis

Pulex irritans

MALDI-TOF MS identification

Cephalothorax

40 μL of Mix: HPLC
Formic
Acetoniic

Molecular identification

A half of dorsal abdomen part

+ 200μL of Buffer + PK

Incubation overnight at 56°C

DNA extraction

Study sites

China

Laos

Thailand

Cambodia

Binh Dinh

Gia Lai

Dak Lak

Dak Nong

Specimens were mounted and confirmed by sequencing

54

Article

Morphological, molecular and MALDI-TOF MS identification of flea species and their associated microorganisms in Vietnam

Ly Na Huynh[1,2,3], Adama Zan Diarra[1,2], Quang Luan Pham[3], Jean-Michel Berenger[1,2], Van Hoang Ho[3], Xuan Quang

Nguyen[3], Philippe Parola[1,2]*

[1] Aix Marseille University, IRD, AP-HM, SSA, VITROME, Marseille, France; lynaimpe@gmail.com (L.N.H); adamazandiarra@gmail.com (A.Z.D)
[2] IHU-Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13005 Marseille, France.
[3] Institute of Malariology, Parasitology and Entomology, Quy Nhon (IMPE-QN), Vietnam; luanimpe@gmail.com (Q.L.P); ho_hoang64@yahoo.com (V.H.H); nxquang134@gmail.com (X.Q.N)
* Correspondence: author :Prof. Philippe Parola : VITROME, Institut Hospitalo-Universitaire Méditerranée Infection, 19-21 Boulevard Jean Moulin 13385 Marseille Cedex 05, France. Phone: + 33 (0) 4 13 73 24 01. Fax: + 33 (0) 4 13 73 24 02. Email: philippe.parola@univ-amu.fr

Abstract: Fleas are obligatory blood-sucking ectoparasites of medical and veterinary importance. The identification of fleas and associated flea-borne microorganisms therefore plays an important role in controlling and managing these vectors. Recently, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has been reported as an innovative and effective approach to the identification of arthropods, including fleas. This study aims to use this technology to identify ethanol-preserved fleas collected in Vietnam and to use molecular biology to search for microorganisms associated with these fleas. A total of 502 fleas were collected from wild and domestic animals in four provinces of Vietnam. Morphological identification led to the recognition of five flea species, namely *Xenopsylla cheopis*, *Xenopsylla astia*, *Pulex irritans*, *Ctenocephalides canis*, and *Ctenocephalides felis*. The cephalothoraxes of 300 individual, randomly selected fleas were tested by MALDI-TOF MS and molecular analysis for identification and microorganism detection. A total of 257/300 (85,7%) of the obtained spectra from cephalothoraxes of each species were of good enough quality to be used for our analyses. Our laboratory MALDI-TOF MS reference database was upgraded with spectra achieved from five randomly selected fleas for every species of *Ctenocephalides canis* and *Ctenocephalides felis*. The remaining spectra were then queried against the upgraded MALDI-TOF MS database, which showed 100% correspondence between morphology and MALDI-TOF MS identification for two flea species (*Ctenocephalides canis* and *Ctenocephalides felis*). The MS spectra of the remaining species (three *P. irritans*, five *X. astia*, and two *X. cheopis*) were visually generated low-intensity MS profiles with high background noise that could not use to update our database. *Bartonella* and *Wolbachia* spp. were detected in 300 fleas from Vietnam by using PCR and sequencing with primers derived from the *gltA* gene for *Bartonella* and *16S* rRNA gene for *Wolbachia*, including three *Bartonella clarridgeiae* (1%), three *Bartonella rochalimae* (1%), one *Bartonella coopersplainsensis* (0.3%), and 174 *Wolbachia* spp. Endosymbiont (58%).

Keywords: MALDI-TOF MS, molecular identification, fleas, flea-borne microorganisms, Vietnam

1. Introduction

Fleas are considered as hosts for a wide range of human pathogens. The most severe infection by fleas is a plague which is caused by the bacterium *Yersina pestis* [1]. Fleas are still known worldwide as important vectors of several other zoonotic pathogens, including *Rickettsia typhi,* the agent of murine typus, and *Bartonella henselae,* the agent of cat-scratch disease (CSD) [2,3].

In Vietnam, *Xenopsylla cheopis* and *Xenopsylla astia* fleas play an important role in the transmission of bubonic plague from rodents to other rodents and to humans [4]. The first two outbreaks of human plague occurred in Nha Trang (1898) and Saigon (1906) (now known as Ho Chi Minh city) [4]. Recently, plague foci have occasionally arisen in the Central Highlands of Vietnam, with 472 confirmed plague cases, leading to 24 deaths [5]. *Pulex irritans* is a vector of various zoonotic pathogens including plague, murine typhus, and *Rickettsia felis* infection [2]. Additionally, *P. irritans* has been described as a potential vector of *B hensaleae*, the agent of CSD and *B. quintana*, the agent of trench fever [6]. Infestation of dogs with the human flea *P. irritans* due to *Bartonella* spp. has been reported in southeast Asia (SEA) [7]. Meanwhile, the *Ctenocephalides* species are ectoparasites with a global distribution and are vectors of various pathogens [8], many of which are also well-known to infect humans [9].

Despite reports of murine typhus, plague, and *rickettsial* and *bartonella* infections [5,10,11], studies on fleas and their associated microorganisms have been poorly investigated in Vietnam [12]. However, there are 51 flea species and subspecies that have been described. Among those, one new species of *Peromyscopsylla himalaica* flea was recently found in Vietnam for the first time [13]. The accurate identification of most fleas is an essential step in studying and surveying flea-borne diseases. Undoubtedly, the list of Vietnamese flea fauna remains incomplete and is likely to be extended if further investigations on fleas are carried out [13]. However, the presence of standard taxonomic keys and reference data specific to Vietnamese flea species are currently lacking, which makes the morphological identification of fleas more difficult and sometimes nigh impossible. The identification of flea species entirely based on morphological aspects that require extensive entomological expertise, therefore, remains challenging for Vietnamese researchers. Over the last two decades, molecular approaches have been used for the identification of arthropods and their associated microorganisms. Nevertheless, there are several drawbacks to these methods, which are time-consuming, expensive, and require primer-specific targeting and reference sequence availability in GenBank.

The development of an alternative tool enabling the rapid, reliable, and affordable identification of fleas is, therefore, needed. Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS), a useful tool that relies on the analysis of protein fingerprints, has revolutionised clinical microbiology diagnostics. More recently, MALDI-TOF MS has successfully been used for the identification of arthropods, including fleas [6]. Specifically, MALDI-TOF MS makes it possible to distinguish between fleas which are infected and not infected by *Borrelia* and *Bartonella* spp. [6]. This study aimed to identify flea species captured from small wild and domestic mammals in Vietnam and their associated microorganisms using morphological, molecular, and MALDI-TOF MS methods.

2. Materials and Methods

2.1. Study sites, flea collection and morphological identification

The collection areas included four provinces: Binh Dinh (13°37′N; 108°59′E) province; Gia Lai (13°11′N; 108°41′E) province; Dak Lak (12°49′N; 108°27′E) province; and Dak Nong (12°40′N; 107°44′E) province, between June and October 2021 in the Central and Highlands areas of Vietnam. The map of Vietnam showing the collection areas was created with QGIS version 3.10 and the Vietnamese layers were downloaded from DIVA-GIS at the following link: https://www.diva-gis.org/datadown (**Figures A1 and B1**). All specimens were collected by an entomological team from IMPE-QN from the skin of rodents and domestic animals (cats and dogs) using forceps. The handling of wild animals in this study was carried out following the guidelines approved by the American Society of Mammalogists (http://www.jstor.org/stable/1383033) [14]. Fleas were morphologically identified first at species level using dichotomous keys [15] by an entomological team from IMPE-QN, Vietnam. Fleas from the same host were counted and then stored in the same tube containing 70% ethanol and placed at room temperature. Flea specimens were then transferred to the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection in Marseille (France) for MALDI-TOF MS and molecular analysis. In the laboratory, all flea specimens were then morphologically verified, again by entomologists, using a magnifying glass (Zeiss Axio Zoom.V16, Zeiss, Marly le Roi, France) and taxonomic keys [15,16]. Morphological identification was performed only if all discriminating characters were

well seen.

Figure 1. (**A**) Map of Vietnam showing the fleas collection sites. (**B**) Field photographs of the sampling collection on different animals (**B**).

2.2. Flea dissection and specimen preparation

Fleas were individually removed from 70% ethanol and rinsed twice in distilled water for five minutes. Each flea was dissected into three parts (cephalothorax, dorsal and ventral half of the abdomen) using a sterile surgical blade and placed in 1.5 ml Eppendorf tubes, as previously described [17]. The cephalothorax of each flea and the dorsal half of

the abdomen were subjected to MALDI-TOF MS and molecular biology analyses, respectively. The ventral halves of the abdomen were frozen at -20 °C for backup.

2.3. DNA extraction and validation of morphological identification by molecular analysis

The dorsal abdomen parts of all fleas were incubated at 56 °C overnight in 180 μL of G2 buffer (Qiagen, Hilden, Germany) and 20 μL of proteinase K (Qiagen, Hilden, Germany). DNA was individually extracted from 200 μL of the incubation solution using an EZ1 DNA tissue kit (Qiagen), according to the manufacturer's recommendations. The eluted DNA extraction was then stored at -20 °C.

DNA samples obtained from fleas selected for molecular identification were submitted to standard PCR in an automated DNA thermal cycle to amplify a 540-base pair (bp) fragment of the mitochondrial *ITS2* gene, as described previously [18]. Amplification of a 1000-bp fragment of the mitochondrial *ITS1* gene [18] was used for all specimens for which we did not obtain a sequence with the *ITS2* gene. DNA from *Ct. felis*, reared in our laboratory, was used as a positive control and a mixture without DNA as a negative control. Purified PCR products were sequenced as previously described [18]. All generated sequences were clustered and analysed using the ChromasPro software (version 1.7.7) (Technelysium Pty. Ltd., Tewantin, Australia), and were then blasted against the reference sequences available in GenBank (http://blast.ncbi.nlm.nih.gov/).

2.4. MALDI-TOF MS analysis

Sample preparation Cephalothoraxes from each flea were individually placed in 1.5-mL Eppendorf tubes and dried at 37 °C overnight. To each tube, 40 μL of high-performance liquid chromatography (HPLC) grade water was added and incubated at 37 °C overnight, as previously described [19]. The cephalothorax was ground up in a mix of 20 μL of 70% (v/v) formic acid (Sigma) and 20 μL of 50% (v/v) acetonitrile (Fluka, Buchs, Switzerland), with a small amount of 1.0 mm diameter glass beads (Sigma, Lyon, France) using a tissue layer machine (Qiagen). The cephalothorax was crushed at a frequency of 30 Hz for one minute three times, as in a previous protocol [20]. After centrifugation, 1 μL of the supernatant of each sample was spotted in quadruplicate onto a MALDI-TOF MS steel plate (Bruker Daltonics, Wissembourg, France) and over-layered after drying at room temperature with a matrix solution composed of 1 μl of saturated alpha-cyano4-hydroxycinnamic acid (Sigma France), 50% acetonitrile (v/v), 2.5% trifluoroacetic acid (v/v) (Sigma-Aldrich Co. Ltd., Gillingham Dorset, UK) and high-performance liquid chromatography (HPLC) grade water, as previously described [21]. The target steel plate was air-dried at room temperature for a few minutes before being deposited into the Microflex LT MALDI-TOF MS apparatus (Bruker Daltonics, Germany) for analysis. The quality of the matrix, sample spotting, and operation of the MALDI-TOF MS machine were administered using the cephalothorax of a *Ct. felis* flea reared in our laboratory as a positive control.

2.5. MALDI-TOF MS parameters

The obtained protein mass profile from the flea cephalothorax was visualised using a Microflex LT MALDI-TOF (Bruker Daltonics, Germany) mass spectrometer with Flex-Control software (version 3.3; Bruker Daltonics), with detection in positive ion linear mode at a laser frequency of 50 Hz in a mass range 2–20 kDa.

2.6. Spectral analysis

The flexAnalysis v.3.3 software was used to evaluate spectral quality, reproducibility, and specificity. Poor quality spectra, i.e. those with low intensity (3000 AU), non-reproducibility, and background noise, were excluded from the study. The reproducibility of MS spectra was determined by comparing the average spectral profiles (MSP, main spectrum profile) obtained from four spots of each flea cephalothorax, according to species, using MALDI-Biotyper v3.0 software (Bruker Daltonics) [22]. The reproducibility and specificity of MS spectra were evaluated using gel-view, principal component analysis (PCA), and cluster analysis (MSP dendrogram). ClinProTools v2.2 with the manufacturer's default settings was used to perform gel-view and PCA. Cluster analysis was carried out based on a comparison of the MSP provided by MALDI-Biotyper v3.0 software with clustering based on protein mass profile (i.e., their mass signals and intensities) [22].

2.7. Reference database creation and blind test

The reference MS spectra were created using spectra from the extracted cephalothoraxes of each flea species using MALDI-Biotyper software v3.0. (Bruker Daltonics) [23]. MSPs were generated using an unbiased algorithm and data from peak position, intensity, and frequency [24]. MS spectra of cephalothoraxes from ten specimens of two flea species (five *Ct. canis* and five *Ct. felis*) identified morphologically and molecularly were added to our homemade MS spectra database [20]. The ten MS spectra of the remaining three flea species (three *P. irritans*, five *X. astia*, and two *X. cheopis*), which were low-quality spectra, were not added to our house MS reference spectra database as reference spectra. A blind test against the updated database was performed with the remaining specimens of both *Ct. canis* and *Ct. felis* flea species (**Table 2**). The log score values (LSVs) obtained from the MALDI-Biotyper software v.3.3, which ranged from 0 to 3, were used to estimate the reliability of species identification.

2.8. PCR detection of microorganisms in fleas

Real-time PCR (quantitative PCR, qPCR) was performed according to the manufacturer's protocol using a PCR detection systema CFX Connect™ Real-Time (Bio-Rad) with the Eurogentec Takyon qPCR kit (Takyon, Eurogentec, Belgium). The qPCR reaction contained 10 µL of Master Mix Roche (Eurogentec, Belgium), 0.5 µL of each primer probe and UDG, 3µL of sterile distilled water and 5 µL of the extracted DNA. DNA from flea specimens was screened to detect microorganisms using specific primers and probes targeting bacteria of the Anaplasmataceae family, *Rickettsia* spp., *Borrelia* spp., *Bartonella* spp., and *Coxiella burnetii* (**Table 1**). DNA positive for bacteria of the Anaplasmataceae family were re-checked using *Wolbachia* spp. specific real-time PCR using the 16S rRNA gene. Negative specimens for *Wolbachia* spp. were then retested by standard PCR using the gene 23S Anaplasmatacea amplifying a 485-bp fragment. The DNA of *Rickettsia montanensis*, *Anaplasma phagocytophilum*, *Bartonella elizabethae*, *Borrelia crocidurae*, and *Coxiella burnetii* was used as a positive control, and DNA from *Ct. felis* from our laboratory which was free of the bacteria tested was used as negative controls. The samples were considered to be positive when the cycle threshold (Ct) was < 36 for all microorganisms tested [25].

Following qPCR, *Bartonella*-positive specimens were subjected to amplifying and sequencing of a 1000-bp fragment of the *glt*A rRNA gene. We randomly selected ten specimens which were *Wolbachia* spp. Positive, following qPCR, and subjected them to amplification and sequencing of a 438-bp fragment of the 16S rRNA gene. The primers and probes used for real-time quantitative and standard PCR in this study are listed in **Table 1.**

Table 1. Sequences of the primer sets used for fleas and flea-borne pathogen detection using qPCR and standard PCR

Microorganisms	Targeted sequence /Amplicon size (bp)	Primers (5'-3') and Probes (Used for qPCR Screening or Sequencing)	References
Anapplasmataceae	*23S*	f_TGACAGCGTACCTTTTGCAT r_GTAACAGGTTCGGTCCTCCA p_6FAM-GGATTAGACCCGAAACCAAG	(26)
	23S (485)	f_ATAAGCTGCGGGGAATTGTC r_TGCAAAAGGTACGCTGTCAC	
Rickettsia spp.	*gltA*(RKND03)	f_GTGAATGAAAGATTACACTATTTAT r_GTATCTTAGCAATCATTCTAATAGC p_6FAM-CTATTATGCTTGCGGCTGTCGGTTC	(27)
Borrelia spp.	*ITS4*	f_GGCTTCGGGTCTACCACATCTA r_CCGGGAGGGGAGTGAAATAG p_TGCAAAAGGCACGCCATCACC	(28)
Bartonella spp.	*ITS2*	f_GATGCCGGGGAAGGTTTTC r_GCCTGGGAGGACTTGAACCT p_GCGCGCGCTTGATAAGCGTG	(29)
	gltA (1000 bp)	f_ACGTCGAAAAGAYAAAAATG r_GTAATRCCAGAAATARAAATC	
Coxiellia burnetii	*IS30A*	f_CGCTGACCTACAGAAATATGTCC r_GGGGTAAGTAAATAATACCTTCTGG p_CATGAAGCGATTTATCAATACGTGTATG	(30)
Wolbachia spp.	*23S* rRNA	Wol-301-f (5'-TGGAACTGAGATACGGTCCAG-3') Wol-478-r (5'-GCACGGAGTTAGCCAGGACT-3') Wol-347-p (6FAM-AATATTGGACAATGGGCGAA)	(31)
	16S rRNA (rrs)	W-Spec_f (5'-CATACCTATTCGAAGGGATAG-3') W-Spec_r (5'-AGCTTCGAGTGAAACCAATTC-3')	(26)

The obtained sequences of *Bartonella* spp. and *Wolbachia* spp. were assembled and analysed using the ChromasPro software (version 1.7.7) (Technelysium Pty. Ltd., Tewantin, Australia), and were then blasted against the NCBI's reference sequences database available in GenBank (http://blast.ncbi.nlm.nih.gov/). The method constructed for phylogenetic tree analysis was the neighbour-joining (NJ) method with 1,000 replicates. DNA sequences were aligned using MEGA software version 7.0 (https://www.megasoftware.net/).

3. Results

3.1. Flea collection and morphological identification

A total of 502 fleas were collected in four provinces of Vietnam including 96 in Binh Dinh, 227 in Gia Lai, 35 in DakLak, and 144 in Dak Nong. Morphologically, the fleas identified belonged to five species (**Figures 2** and **3**), including three *X. cheopis* and eight *X.*

astia collected from rodents, four *P. irritans* from dogs, 51 *Ct. felis* and 436 *Ct. canis* collected from cats and dogs.
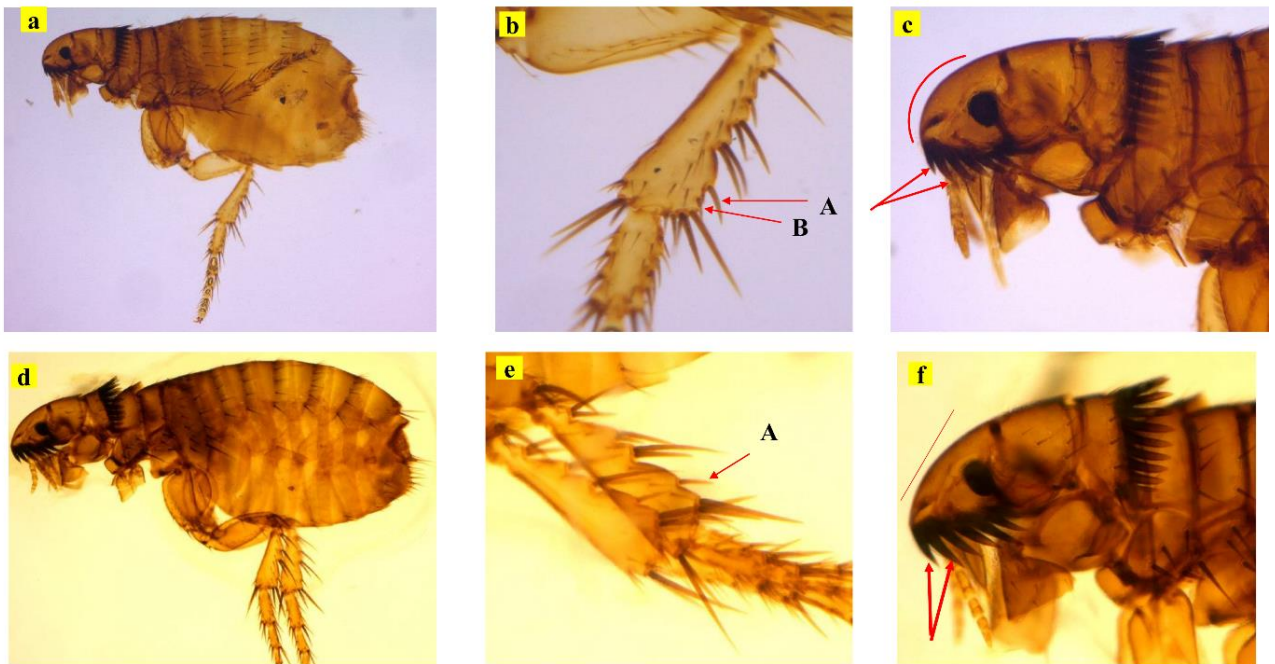

Figure **2.** Morphologically, the 70% ethanol flea-preserved species were collected in Vietnam over a period of between two and six months: ***Ctenocephalides canis*** (♀; a**:** morphological features on an adult flea; **b**: hind tibia with setae A and B; **c**: frons rounded, the first spine of genal comb about half as long as the second); ***Ctenocephalides felis*** (♀; **d**: morphological features on an adult flea; **e**: hind tibia only with setae A; **f**: frons more elongated, the first spine of genal comb only a little shorter than the second).
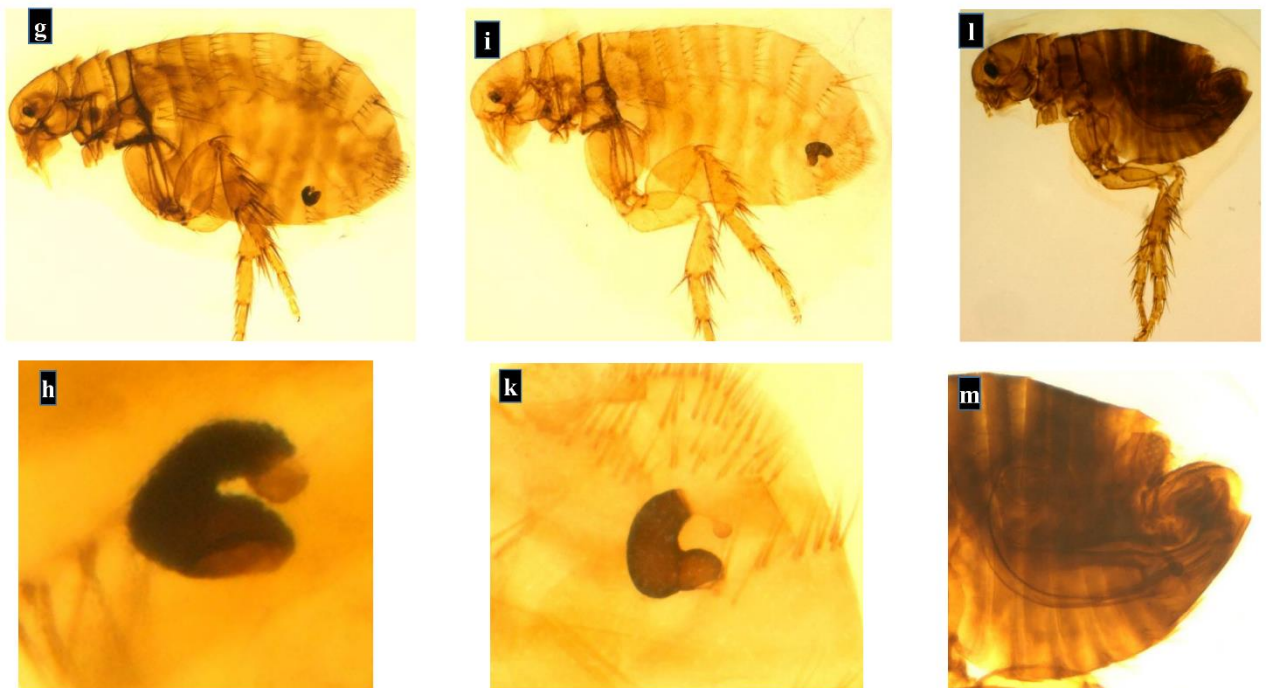

Figure 3. Morphological characters for identification of *Xenopsylla* spp. and *Pulex* sp. ***Xenopsylla cheopis*** (**g**: morphological features on an adult female flea; **h**: spermathecal), ***Xenopsylla astia*** (**i**:

morphological features on an adult female flea; **k**: spermathecal), *Pulex irritans* (**l**: morphological features on an adult male flea; **m**: aedeagus (male sexual organ)

3.2. Molecular identification

To confirm our morphological identification, a total of 20 flea specimens were submitted to molecular analysis using the *ITS*2 gene, including two specimens of *X. cheopis*, five *X. astia*, three *P. irritans*, five *Ct. felis*, and five *Ct. canis* (**Table 2**). We successfully obtained nine sequences of good quality of three flea species using the *ITS2* gene, namely, three specimens for *P. irritans*, five for *X. astia*, and one for *X. cheopis*. The BLAST analysis indicated that fleas morphologically identified as *P. irritans* and *X. cheopis* were 100% identical to their respective homologous sequences available in GenBank (Accession number: KX982861; DQ295095). However, the sequences obtained from five fleas that had been morphologically identified as *X. astia* were more closely related to the sequences deposited in GenBank as *X. cheopis* sequences, with an identity range of 83.7%–84.89% (KX982860). Specimens of two flea species, *Ct. canis* and *Ct felis*, which could not be identified using the *ITS*2 gene were sequenced using the *ITS*1 gene. The BLAST analysis revealed that sequences obtained from *Ct. canis* matched with 100% identity with the GenBank reference sequence for *Ct. canis* (MT895636, HF563590) and those obtained from *Ct. felis* were 99.84%–100% similar to their respective homologous species available in GenBank (MT895636, HF563590).

Table 2. The number of flea species used for MALDI-TOF MS, creation of the MS reference spectra, and molecular biology validation

Morphological identification	Nº tested MALDI-TOF MS/collected	Nº obtained/tested & MolecularID* (%identity; GenBank accession number)	Nº of good spectra/ tested	Nº of spectra added to DB&	MADI-TOF MS ID* (number identified)	LSVs$ [Low-High]
Pulex irritans	3/4	3/3 *Pulex irritans* (99-100%; KX982861)	0/3	0	Not applicable	Not applicable
Xenopsylla cheopis	2/3	1/2 *Xenopsylla cheopis* (100%; DQ295059)	0/2	0	Not applicable	Not applicable
Xenopsylla astia	5/8	5/5 *Xenopsylla cheopis* (83.16-90%; KX982860	0/5	0	Not applicable	Not applicable
Ctenocephalides felis	48/51	5/5 *Ctenocephalides felis* (100%; MT895636, HF583247)	45/48	5	*Ctenocephalides felis* (40)	1.731–2.733
Ctenocephalides canis	242/436	5/5 *Ctenocephalides canis* (100%; MH895642)	212/242	5	*Ctenocephalides canis* (207)	1.708–2.438
5 flea species	300/502		257/300			

3.3. MS spectra analysis

The cephalothoraxes of 300 flea specimens, including three morphologically identified as *P. irritans,* five as *X. astia,* two as *X. cheopis,* 48 as *Ct. felis,* and 242 as *Ct. canis* were randomly selected for MALDI-TOF MS analysis (**Table 2**). Visualisation of MS spectra obtained from *Ct. canis* and *Ct. felis* specimens showed that 93.8% (45/48) and 87.6% (212/242) of the spectra were of high quality (peak intensity > 3,000 arbitrary units (a.u.), no background noise and baseline subtraction correct), respectively (**Figure 4A** and **Table 2**). The intra-species reproducibility and inter-species specificity of the MS spectra of different specimens were confirmed by dendrogram (**Fiure B4**), gel-view, and PCA (**Figures C4**
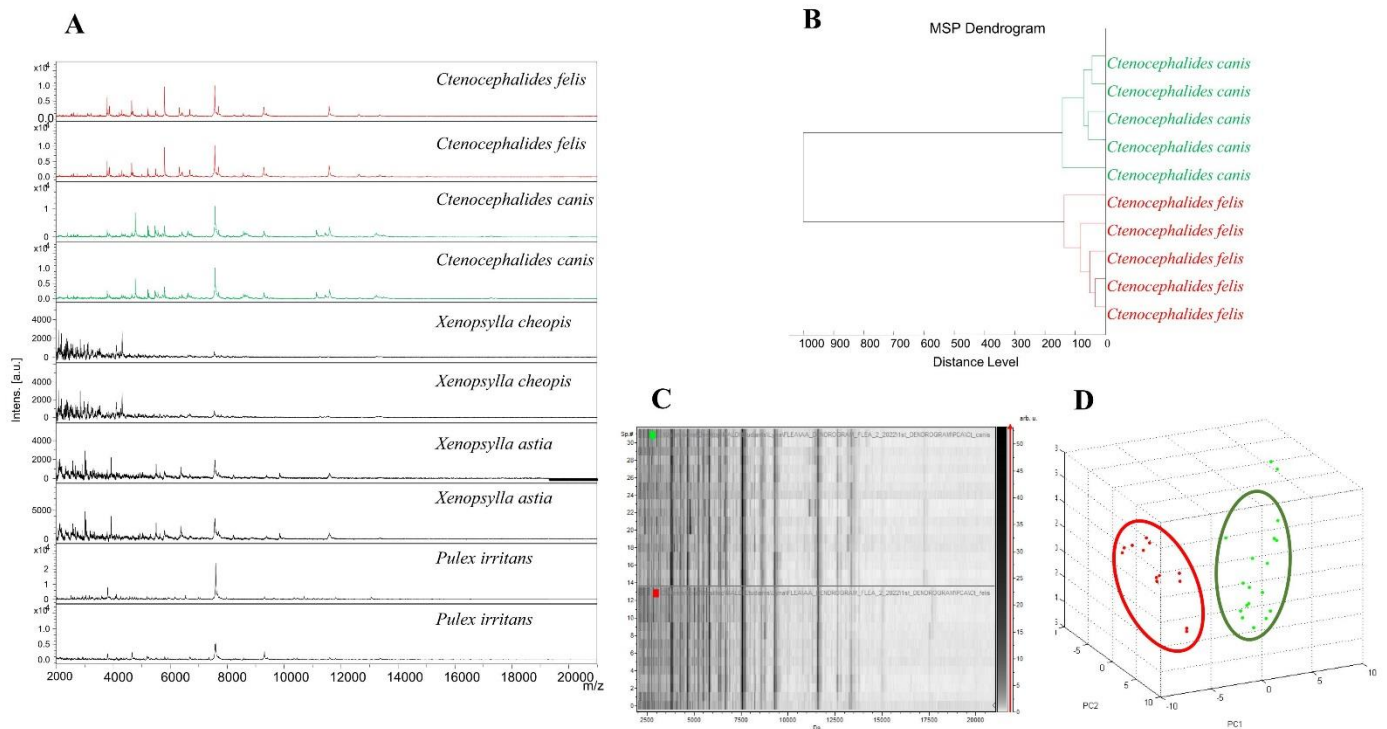
and D**4**) analysis. According to the dendrogram, gel-view, and PCA analysis, all specimens of the same species were on the same branches or were grouped. Conversely, the remaining species' MS spectra were visually generated low-intensity MS profiles with high background noise (**Figure A4**) and hence the spectra from *P. irritans,* *X. astia,* and *X. cheopis* were considered as of insufficient quality and were excluded from the dendrogram, gel-view, and PCA analyses

Figure 4. Comparison of MALDI-TOF MS spectra obtained from different flea species to create the database. Spectral alignment of five flea species showing discriminative peaks using flexAnalysis software; representative MALDI-TOF MS spectra from cephalothorax of *X. cheopis,* *X. astia,* and *P. irritans* with low-intensity MS profiles and high background noise; representative MS spectra of *Ct. canis* and *Ct. felis* with high-quality spectra (peak intensity > 3,000 arbitrary units (a.u.), no background noise with baseline subtracted (**A**). Dendrogram of MALDI-TOF MS spectra of flea species collected in Vietnam. Cluster analysis was performed using Biotyper software v.3.0 (**B**). The MS spectra of different specimens showed intraspecies reproducibility and inter-species specificity as confirmed by PCA (**C** and **D**)

The dendrogram was generated using five representative spectra of each species to evaluate intra-species reproducibility and inter-species specificity revealing the grouping of all flea specimens of the same species on the same branch (**Figure B4**).

3.4. MALDI-TOF MS flea identification

The accuracy of MALDI-TOF MS identification for the flea specimens was assessed by querying 247 specimens (207 *Ct. canis* and 40 *Ct. felis*) morphologically identified against our upgraded MALDI-TOF MS reference database with five spectra per species which was validated by molecular biology (**Table 2).**

Interrogation of the spectra of 247 flea specimens showed that all matched their counterparts in our MALDI-TOF MS database, i.e., a concordance between our morphological identification and MALDI-TOF MS. The LSVs of *Ct. canis* specimens ranged from 1.708–2.438 (mean: 2.007 ± 0.158), and of those of *Ct. felis* ranged from 1.731–2.733 (mean: 2.183 ± 0.171). The spectra of the flea specimens updated in the MS protein profile database were deposited on the website of the University Hospital Institute (UHI) under the following DOI: https://doi.org/10.35088/rbqp-g648.

3.5. Detection of microorganisms in fleas

The DNA of a total of 300 fleas was screened for five bacterial groups (bacteria of Anaplasmataceae family, *Rickettsia* spp., *Borrelia* spp., *Bartonella* spp., and *Coxiella burnetii*) using qPCR. Only DNA of bacteria of the Anaplasmataceae family and *Bartonella* spp. were detected in 184/300 (60.3%) of our specimens. DNA of bacteria of the Anaplasmataceae family was detected in 174/300 (58%) of fleas (**Table 3).** DNA of *Bartonella* spp. was detected in 10/300 (3%) fleas by qPCR using the *ITS2* gene (**Table 3**). Infected specimens included *Ct. canis*, *Ct felis*, and *X. astia*. Notably, five *X. astia* and one *Ct. canis* were co-infected with both Anaplasmataceae and *Bartonella* spp. DNA of bacteria of *Rickettsia* spp., *Borrelia* spp., and *Coxiella burnetii* was not found in any fleas.

Table 3. Microorganisms detected using real-time PCR in fleas

Microorganisms tested	Flea species			
	X. astia	*Ct. canis*	*Ct. felis*	Total (%)
Anaplasmataceae	5	148	21	174 (58%)
Bartonella **spp.**	4	10	-	7 (2.3%)
Rickettsia **spp.**	-	-	-	-
Borrelia **spp.**	-	-	-	-
Coxiella burnetii	-	-	-	-

All 174 flea specimens that were positive for the Anaplasmataceae family were then found to be positive for *Wolbachia* spp. using the *16S* rRNA gene. We randomly selected ten positive *Wolbachia* specimens for sequencing. The analysis of the *Wolbachia 16S* rRNA fragment indicated that *Wolbachia* spp. from *X. astia*, *Ct. felis*, and *Ct. canis* were between 99.69% and 100% similar to the corresponding sequences of *Wolbachia pipientis* (MN123077, MN123078) as well as other sequences deposited in GenBank as *Wolbachia endosymbionts* of various arthropods (DQ399344). A total of seven out of ten *Bartonella*-positive specimens were successfully amplified by standard PCR targeting a fragment of the *gltA* gene, the BLAST analysis showed genetic distinctions in the three species of *Bartonella* spp. found in our study based on the *glt*A gene. Specifically, the BLAST analysis of three sequences obtained from *Ct. canis* was between 99.2% and 100% identical to the corresponding sequences of *Bartonella clarridgeiae* (KJ170239; KY913636), and three sequences

achieved from *X. astia* were 100% similar with the corresponding sequences of *Bartonella rochalimae* (FN645459). One sequence obtained from *X. astia* was 100% identical to the corresponding sequence of *Bartonella coopesplainersis* (EU111803) (**Table 4).**

Table 4. Microorganisms detected using sequencing in fleas

Microorganisms tested	Per ident (%)	Flea species			
		X. astia	*Ct. canis*	*Ct. felis*	Total (%)
Wolbachia endosymbiont		3		2	NA
Wolbachia pipiens	99.69 - 100		5	-	NA
Bartonella clarridgeiae	99.2 - 100	-	3	-	3 (1%)
Bartonella rochalimae	100	3	-	-	3 (1%)
Bartonella coopersplainsensis	100	1	-	-	1 (0.3%)

[1] NA; Not applicable

Two phylogenetic tree analyses of *Wolbachia* spp. and *Bartonella* spp. were generated from the *16S* rRNA and *gltA* genes sequences of our amplicons, respectively. These phylogenetic trees indicated that the detected microorganisms are close to their homologues available in GenBank (**Figures 5** and **6**).
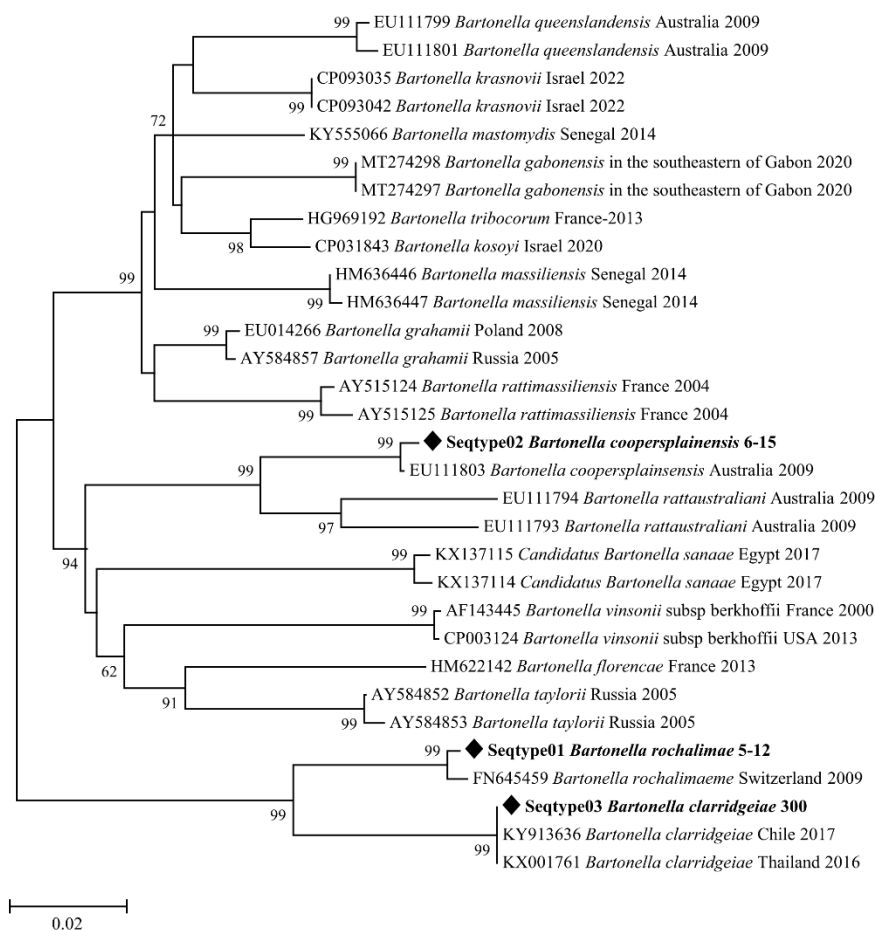
Figure 5. Neighbour-joining (NJ; 500 bootstrap replicates) phylogenetic tree of the *gltA* gene. *Bartonella* spp. sequences (◆) obtained in this study
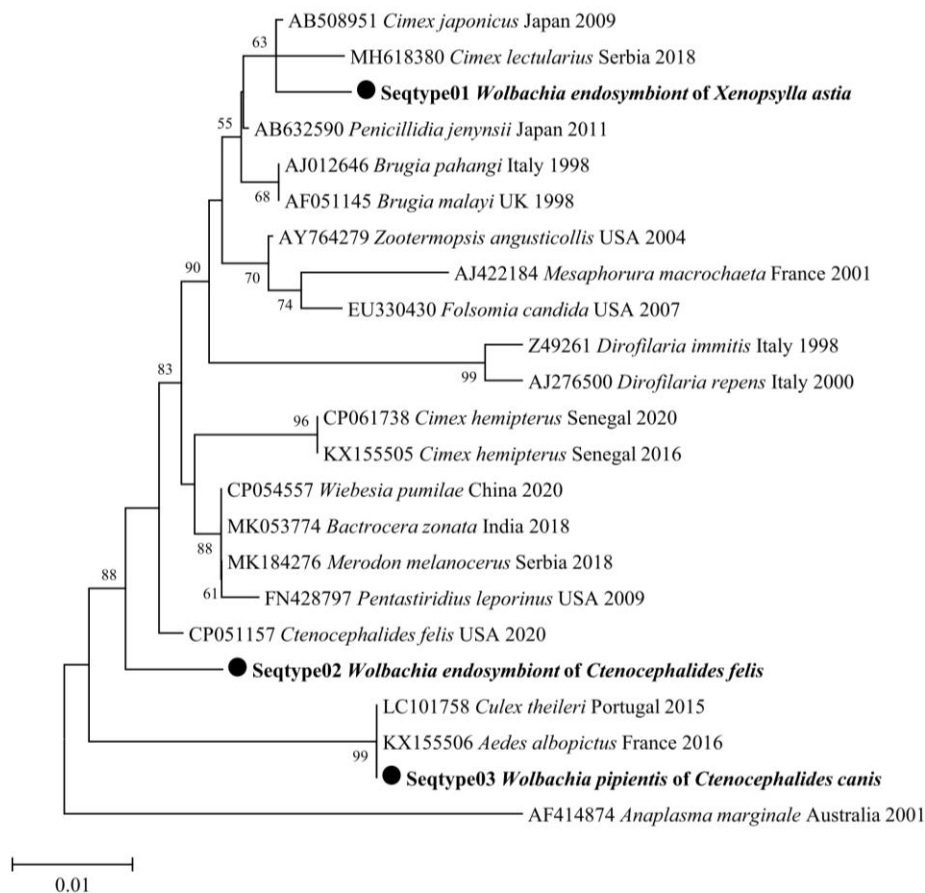

Figure 6. Neighbour-joining (NJ; 500 bootstrap replicates) phylogenetic tree of the *16S rRNA* gene. *Wolbachia* spp. (●) collected from *Xenopsylla astia*, *Ct. felis*, and *Ct. canis*. *Wolbachia* strains are designated following the names of their host species

Discussion

The fleas analysed in our study were collected mainly from cats, dogs and small wild rodents. These ectoparasites are well known to be vectors of human and veterinary pathogens, which are considered important for public health worldwide.

In this study, the morphological identification of fleas collected in Vietnam revealed the presence of five species, namely, *X. astia*, *X. cheopis*, *P. irritans*, *Ct. canis*, and *Ct. felis*. All these species had already been recorded in Vietnam and several countries in southeast Asia (SEA), including Laos, Thailand, and Malaysia [32–37]. *X. cheopis* was the species most predominantly captured from wild rodents in Vietnam [5]. *X. cheopis* is best known for being a vector in the transmission of *Y. pestis*, the bacterial agent of the bubonic plague primarily responsible for two pandemics that marked human history in Vietnam [4] and which still poses a threat to public health in endemic countries [38–42]. *X. astia* is also included here because it has been identified in other plague hit countries (43–45). In Vietnam, the presence of *X. cheopis* and *X. astia* plague vectors co-exist and parasitise in

commensal rodents living inside or outside dwellings but also in open biotopes (agricultural areas, savanna grasslands) and forests [33].

Ctenocephalides canis was the dominant flea species infection in 86.9% of both dogs and cats, followed by 10.2% *Ct. felis*, as already reported worldwide, including SEA [7,46]. These species are known as competent vectors of zoonotic pathogens such as *Rickettsia felis* and *Bartonella* spp. [47]. *Ct. felis* is the most well-known vector of *Rickettsia felis*, a causative agent of spotted fever group rickettsiosis [48]. In SEA, the first human case of *R. felis* infection was identified on Thai-Myanmar border, as described by Parola *et al.* [49]. Since then, many human infections have been reported in Laos [50], Indonesia [51], and Vietnam [10]. Several studies have shown *R. felis* in *Ct. felis* flea in Vietnam [7,12]. However, no *Rickettsia*-positive fleas were found in our specimens. Similarly, no *C. felis* analysed showed evidence of the carriage of *Bartonella* bacteria in our fleas. In contrast, we reported that three *Ct. canis* dog fleas were found to be infected with the *B. clarridgeiae* species, which is the causative agent of CSD in humans. This bacterium was the most common species found in cat fleas and was also detected in humans, cats, and dogs [52,53]. The human flea *P. irritans* was collected from 0.8% (4/502) of dogs in our study. *P. irritans* is widespread globally and has also been detected in other wild animals (birds, rodents, bats, carnivores, and ungulates [54,55]. Furthermore, this flea plays a role in domestic human-to-human transmission of *Y. pestis* [54,56]. However, it has also been identified as a vector of several bacterial pathogens, such as *Rickettsia* and *Bartonella* species [57]. Nevertheless, no evidence of *Bartonella* was found from *P. irritans* fleas in our study.

Molecular biology was used to confirm the morphological identification of five flea species collected in Vietnam, namely, *X. cheopis*, *X. astia*, *P. irritans*, *Ct. canis*, and *Ct. felis* using either the *ITS1* or *ITS2* gene, homologous sequences of which were available in GenBank. The *ITS1* gene was chosen to differentiate *Ct. felis* from *Ct. canis* because this specific marker shows unique divergence compared with other genes frequently used to identify arthropods [58]. Nevertheless, the reference DNA barcode sequences deposited in GenBank miss the *ITS1* region or have only recently been updated [58,59], which shows the limited range of molecular technology in the choice of targeted sequences. Furthermore, one species, morphologically identified as *X. astia*, did not match the molecular identification result. This discrepancy was due to the lack of DNA sequence information for *X. astis* species in the GenBank database, which is also one of the described drawbacks of molecular biology [60,61].

MALDI-TOF MS has revolutionised clinical microbiology diagnostics as a result of its advantages in the routine identification of bacteria, archaea, and fungi [62]. Recently, MALDI-TOF MS has emerged as an efficient approach to the rapid and accurate identification of arthropod vectors, including fleas [6,20,59]. In our study, the high-quality spectra of *Ct. canis* and *Ct. felis* were 87.6 % and 93.8%, respectively. Unfortunately, it was not possible to get good quality spectra from three flea species (corresponding to three *P. irritans* samples from dogs, five *X. astia*, and two *X. cheopis* from rodents) in order to update the database and then identify using a blind test. The theories were excluded from our results, for example: (1) the specimens were stored in ethanol for a long time, while all our samples were preserved and subsequently analysed for a period of between two and six months. Ethanol is widely used for the conservation and transportation of arthropods under field conditions because it is less restrictive than freezing [63]. Several studies have shown the efficacy of MALDI-TOF MS for the identification of arthropods preserved in ethanol for between two and ten years [61,63] and even up to several decades [19]; (2) the choice of the compartment to be performed for MALDI-TOF MS, cephalothorax, chosen

for the MALDI-TOF MS analysis, based on previous data indicating that this body part has high-quality spectra compared to different parts of the body [6,17,20]. The limited sample size for each species (between two and five specimens per species) may explain our spectra results.

We report, for the first time, the presence of *Wolbachia* spp. in three flea species from Vietnam. *Wolbachia* is a genus of bacterial endosymbiont that is known to infect both nematodes and arthropods [64]. These bacteria have been reported to enable transmission from parents to their descendants and may alter the biology, ecology, and evolution of its hosts by acting on feminisation, parthenogenesis, male-killing in the arthropods, and hence cause the cytoplasmic incompatibility of spermatozoa [64]. In Vietnam, the molecular detection of *Wolbachia* in natural mosquito populations and other arthropods has not been recorded. However, information on the detection of *Wolbachia* spp. on arthropods, including fleas found in the tropical regions of Laos, Thailand, and Malaysia, has been published [65]. In our study, 58% of the fleas were positive for *Wolbachia* spp. endosymbiont DNA. The prevalence of *Wolbachia* found in our work is higher than that identified in a study of cat fleas in France [29], although similar results on the occurrence of the *Wolbachia* infection has been established in Malaysia [65].

Bartonella species are intracellular parasites of erythrocytes of a wide range of various mammalian and ectoparasite hosts [29]. Most of these bacterial species have been detected in their arthropod vectors, including fleas, ticks, and lice [6,67,68], although the majority of these arthropods' roles as vectors has yet to be proven [69]. Herein, we showed the presence of two human pathogens of the *Bartonella* genus (*B. clarridgeiae* and *B. rochalimae*) [70] and one *B. coopersplainsensis* in an endemic Australian rat [71]. The occurrence of *Bartonella* from rodent and cat fleas has been reported in China [70], Thailand [72], Japan [73], Nepal [74], Indonesia [75], and Cambodia [76]. Our results provide the first molecular evidence of *Bartonella clarridgeiae*, *B. coopersplainsensis*, and *B. rochalimae* in rodent and dog fleas from Vietnam and suggest their related flea-borne diseases.

Bartonella clarridgeiae has been suspected to be an additional agent of CSD, and its pathogenic role in humans has been demonstrated in Ireland [77] and the United States [78]. *B. clarridgeiae* is also known as a veterinary pathogen associated with disease in cats and dogs [79]. *Bartonella* spp. (*B. elizabethae*, *B. rattimassiliensis*, *B. tribocorum*, *B. coopersplainensis*, and *B. queenslandensis*) have already been reported from rat mites in the Mekong Delta [80] and blood samples from rodents and bats in Vietnam [11,81]. However, the *Bartonella* genus, especially, *B. clarridgeiae* was first discovered in *Ct. canis* dog fleas in our study. It has been previously been found in fleas in southeast Asia (SEA) from the Thai-Myanmar border [82], Laos [32], the Philippines [83], and Indonesia [84].

We found that 1% of *X. astia* fleas, which parasitise small wild rodent species, harbour *B. rochalimae* DNA by sequence analysis based on the *gltA* gene. *B. rochalimae* is the causative agent of CSD, which can be transmitted to humans by fleas and mites [85]. Several cases of human infection have been described in the United States [86]. A *B. rochalimae* infection was first reported in a dog from California [87]. Wild carnivores such as coyotes, foxes, and skunks, have been suggested to be major reservoirs for *B. rochalimae* in nature [85]. In Asia, *B. rochalimae* has been found in wild rodents near the China-Kazakhstan border [70].

We showed, for the first time, the prevalence of *B. coopersplainsensis* in *X. astia* rodent fleas captured in Vietnam. This *Bartonella* bacterium has not yet been described in humans [66]. However, *B. coopersplainsensis* has been detected in rats collected from SEA [76,88,89],

Brazil [66], and New Zealand [90], and from other wild rodents in China [91] and Lithuania [92]. In Vietnam, *B. coopersplainsensis* has already been detected in trombiculid mites of rats and their reservoir host's blood samples [80].

The *Bartonella* species detected in our specimens were *B. clarridgeiae*, *B. rochalimae*, and *B. coopersplainensis*, with no evidence of other species such as *Bartonella* spp. (*B. rattimassiliensis*, *B. tribocolum*), being reported in the SEA region. Our results demonstrate that *X. astia* and *Ct. canis* fleas are potential vectors of *B. clarridgeiae* and *B. rochalimae*, which might play role in human infection in the Central Highlands of Vietnam.

Conclusion

Our work proves that MALDI-TOF MS as an efficient tool for the rapid and accurate identification of the ethanol-stored flea species. It has also provided evidence of dogs and rodent fleas being the vectors for carrying *Bartonella* species in Vietnam. The detection of these pathogenic bacteria in their ectoparasites may be applied to epidemiologic surveillance and prevention strategies. Hence, further studies will be needed to identify the potential factors of *B. clarridgeiae*, *B. rochalimae*, and *B. coopersplainsensis* and to investigate whether small wild rodents and domestic dogs, as well as ectoparasite vectors, are of zoonotic importance.

Ethics statement: The study was approved by the research ethics committee of the Institute of Malariology, Parasitology, and Entomology, Quy Nhon (IMPE-QN) on behalf of the Vietnamese Ministry of Health (Approval No. 380/CT-VSR-2021). Permission was obtained from local authorities for wild mammals that were not listed in national parks or other protected areas in Vietnam. For dogs and cats, permission was obtained from the owners. Rodents were captured using live animal traps and released into their natural habitat after collecting flea samples.

References

1. Barbieri, R.; Signoli, M.; Chevé, D.; Costedoat, C.; Tzortzis, S.; Aboudharam, G.; *et al*. *Yersinia pestis*: the Natural History of Plague. *Clin Microbiol Rev*. 2020 Dec 16;34(1):e00044-19.

2. Bitam, I.; Dittmar, K.; Parola, P.; Whiting, M.F.; Raoult, D. Fleas and flea-borne diseases. *International Journal of Infectious Diseases*. 2010 Aug;14(8):e667–76.

3. Hamzaoui, B.E.; Zurita, A.; Cutillas, C.; Parola, P. Fleas and flea-borne diseases of North Africa. *Acta Trop*. 2020 Nov;211:105627.

4. Cavanaugh, D.C.; Dangerfield, H.G.; Hunter, D.H.; Joy, R.J.; Marshall, J.D.; Quy, D.V.; *et al*. Some observations on the current plague outbreak in the Republic of Vietnam. *Am J Public Health Nations Health*. 1968 Apr;58(4):742–52.

5. Pham, H.V.; Dang, D.T.; Tran, M.N.N.; Nguyen, N.D.; Nguyen, T.V. Correlates of environmental factors and human plague: an ecological study in Vietnam. *Int J Epidemiol*. 2009 Dec;38(6):1634–41.

6. El Hamzaoui, B.; Laroche, M; Almeras, L.; Bérenger, J.M.; Raoult, D.; Parola, P. Detection of *Bartonella* spp. in fleas by MALDI-TOF MS. *PLoS Negl Trop Dis*. 2018 Feb 16;12(2):e0006189.

7. Nguyen, V.L.; Colella, V.; Greco, G.; Fang, F.; Nurcahyo, W.; Hadi, U.K.; *et al*. Molecular detection of pathogens in ticks and fleas collected from companion dogs and cats in East and Southeast Asia. *Parasit Vectors*. 2020 Aug 15;13(1):420.

8. Álvarez-Fernández, A.; Breitschwerdt, E.B.; Solano-Gallego, L. *Bartonella* infections in cats and dogs including zoonotic aspects. *Parasit Vectors*. 2018 Dec 4;11:624.

9. Beugnet, F.; Marié, J.L. Emerging arthropod-borne diseases of companion animals in Europe. *Veterinary Parasitology*. 2009 Aug 26;163(4):298–305.

10. Le-Viet, N.; Le ,V.N.; Chung, H.; Phan, D.T.; Phan, Q.D.; Cao, T.V.; *et al*. Prospective case-control analysis of the aetiologies of acute undifferentiated fever in Vietnam. *Emerg Microbes Infect*. 2019 Mar 4;8(1):339–52.

11. Anh, L.T.L.; Balakirev, A.E.; Chau, N.V. Investigation of multiple infections with zoonotic pathogens of rodents in northern Vietnam. *J Vector Borne Dis*. 2021 Mar;58(1):47–53.

12. Do, T.; Inpankaew, T.; Duong, D.H; Bui, K.L. First Molecular Evidence of Pathogens in Fleas Collected from Dogs in Northern Vietnam. *Pathogens*. 2021 Sep 14;10(9):1185.

13. Porshakov,. AM.; Korneev, M.G.; Chumachkova, E.A; Chau, N.V.; Toan, T.V.; Cuong, V.V. Addition to the Flea Fauna of Vietnam. *Entomological Review*. 2021 Dec 1;101(9):1287–92.

14. Kirkland, G.L. Guidelines for the Capture, Handling, and Care of Mammals as Approved by the American Society of Mammalogists. *Journal of Mammalogy*. 1998 Dec 3;79(4):1416–31.

15. Pratt, H.D. Flea diagram - with structures labeled. :8.

16. Beaucournu, J.C.; Launay, H. Les Puces de la France et du Bassin M ´editerran ´een Occidental. F´ed ´eration Française des Soci ´et ´es de Sciences Naturelles:; ISBN 2-903052-10-7, France. 1990.

17. Yssouf, A.; Socolovschi, C.; Leulmi, H; Kernif, T.; Bitam, I.; Audoly, G.; *et al*. Identification of flea species using MALDI-TOF/MS. *Comp Immunol Microbiol Infect Dis*. 2014 May;37(3):153–7.

18. Vobis, M.; D'Haese, J.; Mehlhorn, H.; Mencke, N.; Blagburn, B.L.; Bond, R.; *et al*. Molecular phylogeny of isolates of *Ctenocephalides felis* and related species based on analysis of ITS1, ITS2 and mitochondrial 16S rDNA sequences and random binding primers. *Parasitol Res*. 2004 Oct;94(3):219–26.

19. Ahamada, M.S.; Diarra, A.Z.; Almeras, L.; Parola, P. Identification of ticks from an old collection by MALDI-TOF MS. *J Proteomics*. 2022 Jul 30;264:104623.

20. Nebbak, A.; El Hamzaoui, B.; Berenger, J.M.; Bitam, I.; Raoult, D.; Almeras, L.; *et al*. Comparative analysis of storage conditions and homogenization methods for tick and flea species for identification by MALDI-TOF MS. *Med Vet Entomol*. 2017 Dec;31(4):438–48.

21. Yssouf, A.; Almeras, L.; Raoult, D.; Parola, P. Emerging tools for identification of arthropod vectors. *Future Microbiol*. 2016;11(4):549–66.

22. Nebbak, A.; Willcox, A.C.; Bitam, I.; Raoult, D.; Parola, P.; Almeras, L. Standardization of sample homogenization for mosquito identification using an innovative proteomic tool based on protein profiling. *Proteomics*. 2016 Dec;16(24):3148–60.

23. Yssouf, A.; Parola, P.; Lindström, A.; Lilja, T.; L'Ambert, G.; Bondesson, U.; *et al*. Identification of European mosquito species by MALDI-TOF MS. *Parasitol Res*. 2014 Jun;113(6):2375–8.

24. Vega-Rúa, A.; Pagès, N.; Fontaine, A.; Nuccio, C.; Hery, L.; Goindin, D.; *et al*. Improvement of mosquito identification by MALDI-TOF MS biotyping using protein signatures from two body parts. *Parasit Vectors*. 2018 Nov 3;11:574.

25. Lafri, I.; El Hamzaoui, B.; Bitam, I.; Leulmi, H.; Lalout, R.; Mediannikov, O.; *et al*. Detection of relapsing fever *Borrelia* spp., *Bartonella* spp. and Anaplasmataceae bacteria in argasid ticks in Algeria. *PLoS Negl Trop Dis*. 2017 Nov;11(11):e0006064.

26. Djiba, M.L.; Mediannikov, O.; Mbengue, M.; Thiongane, Y.; Molez, J.F.; Seck, M.T.; *et al*. Survey of Anaplasmataceae bacteria in sheep from Senegal. *Trop Anim Health Prod*. 2013 Oct;45(7):1557–61.

27. Rolain, J.M.; Stuhl, L.; Maurin, M.; Raoult, D. Evaluation of antibiotic susceptibilities of three rickettsial species including *Rickettsia felis* by a quantitative PCR DNA assay. *Antimicrob Agents Chemother*. 2002 Sep;46(9):2747–51.

28. Mediannikov, O.; Trape, J.F.; Diatta, G.; Parola, P.; Fournier, P.E.; Raoult, D. *Rickettsia africae*, Western Africa. *Emerg Infect Dis*. 2010 Mar;16(3):571–3.

29. Rolain, J.M.; Franc, M.; Davoust, B.; Raoult, D. Molecular Detection of *Bartonella quintana*, *B. koehlerae*, *B. henselae*, *B. clarridgeiae*, *Rickettsia felis*, and *Wolbachia pipientis* in Cat Fleas, France. *Emerg Infect Dis*. 2003 Mar;9(3):339–42.

30. Rolain, J.M.; Raoult, D. Molecular detection of *Coxiella burnetii* in blood and sera during Q fever. *QJM*. 2005 Aug;98(8):615–7; author reply 617-620.

31. Laidoudi, Y.; Levasseur, A.; Medkour, H.; Maaloum, M.; Ben, K.M.; Sambou, M.; *et al*. An Earliest Endosymbiont, *Wolbachia massiliensis* sp. nov., Strain PL13 from the Bed Bug (*Cimex hemipterus*), Type Strain of a New Supergroup T. *Int J Mol Sci*. 2020 Oct 29;21(21):8064.

32. Nguyen, H.M.; Theppannga, W.; Vongphayloth, K.; Douangngeun, B.; Blacksell, S.D.; Robinson, M.T. Screening of ectoparasites from domesticated dogs for bacterial pathogens in Vientiane, Lao PDR. *Zoonoses Public Health*. 2020 Dec;67(8):862–8.

33. Suntsov, V.V.; Huong, L.T.; Suntsova, N.I.; Gratz, N.G. Plague foci in Viet Nam: zoological and parasitological aspects. *Bull World Health Organ*. 1997;75(2):117–23.

34. Suntsov, V.V.; Huong, L.T.; Suntsova, N.I. [Notes on fleas (Siphonaptera) in plague foci on the Tay Nguyen plateau (Vietnam)]. *Parazitologiia*. 1992 Dec;26(6):516–20.

35. Kernif, T.; Socolovschi, C.; Wells, K.; Lakim, M.B.; Inthalad, S.; Slesak, G.; *et al*. *Bartonella* and *Rickettsia* in arthropods from the Lao PDR and from Borneo, Malaysia. *Comp Immunol Microbiol Infect Dis*. 2012 Jan;35(1):51–7.

36. Beaucournu, J.C.; Sountsova, N.I.; Ly, T.V.H.; Sountsov, V.V. Contribution à l'étude de la peste au Vietnam : historique et inventaire des puces signalées (Insecta - Siphonaptera) en zones anthropisées. *Parasite*. 2002 Mar;9(1):3–10.

37. Le, T.D.; Tran, N.H.; Tran, T.K.T.; Mai, D.T.; Doan, B.M. Research on species composition and distribution of some external parasites groups in Southern – Lam Dong area. Available online: http://www.tapchiyhocduphong.vn/tap-chi-y-hoc-du-phong/2015/08/nghien-cuu-thanh-phan-loai-va-phan-bo-mot-so-nhom-ngoai-ky-sinh-tai-mot-so-diem--o81E20300.html (accessed on 21 Jun 2022).

38. Porshakov, A.; Чумачкова, А.; Касьян, Ж.; Оглодин.; Мо, Т.; Кыонг, В.; *et al*. Results of Epizootiological Survey on Plague and Other Zoonotic Infections in the Northern Provinces of the Socialist Republic of Vietnam During Spring Months of 2019. 2020 Apr 24;

39. Andrianaivoarimanana, V.; Kreppel, K.; Elissa, N.; Duplantier, J.M; Carniel, E.; Rajerison, M.; *et al*. Understanding the persistence of plague foci in Madagascar. *PLoS Negl Trop Dis*. 2013 Nov;7(11):e2382.

40. Eisen, R.J.; Atiku, L.A.; Enscore, R.E.; Mpanga, J.T.; Acayo, S.; Mead, P.S.; *et al*. Epidemiology, Ecology and Prevention of Plague in the West Nile Region of Uganda: The Value of Long-Term Field Studies. *Am J Trop Med Hyg*. 2021 Jul;105(1):18–23.

41. Crook, L.D.; Tempest, B. Plague. A clinical review of 27 cases. *Arch Intern Med*. 1992 Jun;152(6):1253–6.

42. He, Z.; Wei, B.; Zhang, Y.; Liu, J.; Xi, J.; Ciren, D.; *et al*. Distribution and Characteristics of Human Plague Cases and *Yersinia pestis* Isolates from 4 Marmota Plague Foci, China, 1950-2019. *Emerg Infect Dis*. 2021 Oct;27(10):2544–53.

43. Goyle, A.N. On the Transmission of Plague by *Xenopsylla astia* and *X. cheopis*. Preliminary Observations *Being a paper read at the Indian Science Congress, Lahore, in January, 1927. *Ind Med Gaz*. 1927 Jun;62(6):317–8.

44. Tun, M.M.; King, R.E. The host/parasite relationships of *Xenopsylla astia* and *X. cheopis* on *Bandicota bengalensis* in Rangoon, Burma. *Southeast Asian J Trop Med Public Health*. 1979 Dec;10(4):505–9.

45. Kia, E.; Moghddas-Sani, H.; Hassanpoor, H; Vatandoost, H.; Zahabiun, F.; Akhavan, A.; *et al*. Ectoparasites of rodents captured in bandar abbas, southern iran. *Iran J Arthropod Borne Dis*. 2009;3(2):44–9.

46. Zarea, A.A.K.; Bezerra-Santos, M.A.; Nguyen, V.L.; Colella, V.; Dantas-Torres, F.; Halos, L.; *et al*. Occurrence and bacterial loads of *Bartonella* and haemotropic *Mycoplasma* species in privately owned cats and dogs and their fleas from East and Southeast Asia. *Zoonoses Public Health*. 2022 May 11;

47. Lawrence, A.L.; Hii, S.F.; Jirsová, D.; Panáková, L.; Ionică, A.M.; Gilchrist, K.; *et al*. Integrated morphological and molecular identification of cat fleas (*Ctenocephalides felis*) and dog fleas (*Ctenocephalides canis*) vectoring *Rickettsia felis* in central Europe. *Veterinary Parasitology*. 2015 Jun;210(3–4):215–23.

48. Parola, P.; Davoust, B.; Raoult, D. Tick- and flea-borne rickettsial emerging zoonoses. *Vet Res*. 2005 May;36(3):469–92.

49. Parola, P.; Cornet, J.P.; Sanogo, Y.O.; Miller, R.S.; Thien, H.V.; Gonzalez, J.P.; *et al*. Detection of *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., and other eubacteria in ticks from the Thai-Myanmar border and Vietnam. *J Clin Microbiol*. 2003 Apr;41(4):1600–8.

50. Dittrich, S.; Phommasone, K.; Anantatat, T.; Panyanivong, P.; Slesak, G.; Blacksell, S.D.; *et al*. *Rickettsia felis* Infections and comorbid conditions, Laos, 2003-2011. *Emerg Infect Dis*. 2014 Aug;20(8):1402–4.

51. Mawuntu, A.H.P.; Johar, E.; Anggraeni, R.; Feliana, F.; Bernadus, J.B.B.; Safari, D.; *et al*. *Rickettsia felis* identified in two fatal cases of acute meningoencephalitis. *PLoS Negl Trop Dis*. 2020 Feb;14(2):e0007893.

52. Mokhtar, A.S.; Tay, S.T. Molecular Detection of *Rickettsia felis*, *Bartonella henselae*, and *B. clarridgeiae* in Fleas from Domestic Dogs and Cats in Malaysia. *Am J Trop Med Hyg*. 2011 Nov 1;85(5):931–3.

53. Abreu-Yanes, E.; Abreu-Acosta, N.; Kosoy, M.; Foronda, P. Molecular detection of *Bartonella henselae*, *Bartonella clarridgeiae* and *Rickettsia felis* in cat and dog fleas in Tenerife, Canary Islands, Spain. *Journal of Vector Ecology*. 2020 Dec;45(2):233–40.

54. Miarinjara, A.; Bland, D.M.; Belthoff, J.R.; Hinnebusch, B.J. Poor vector competence of the human flea, *Pulex irritans*, to transmit *Yersinia pestis*. *Parasit Vectors*. 2021 Jun 10;14:317.

55. Hornok, S.; Beck, R.; Farkas, R.; Grima, A.; Otranto, D.; Kontschán, J.; *et al*. High mitochondrial sequence divergence in synanthropic flea species (Insecta: Siphonaptera) from Europe and the Mediterranean. *Parasit Vectors*. 2018 Apr 2;11:221.

56. Ratovonjato, J.; Rajerison, M.; Rahelinirina, S.; Boyer, S. *Yersinia pestis* in *Pulex irritans* Fleas during Plague Outbreak, Madagascar. *Emerg Infect Dis*. 2014 Aug;20(8):1414–5.

57. O'Donnell, M.; Elston, D.M. What's Eating You? Human Flea (*Pulex irritans*). 2020;106(5). Available online: https://www.mdedge.com/dermatology/article/231222/infectious-diseases/whats-eating-you-human-flea-pulex-irritans (accessed on 5 Aug 2022).

58. Zurita, A.; Callejón, R.; De Rojas, M.; Gómez López, M.S.; Cutillas, C. Molecular study of *Stenoponia tripectinata tripectinata* (Siphonaptera: Ctenophthalmidae: Stenoponiinae) from the Canary Islands: Taxonomy and phylogeny. *Bull Entomol Res*. 2015 Dec;105(6):704–11.

59. Zurita, A.; Gutiérrez, S.G.; Cutillas, C. Infection Rates of *Wolbachia* sp. and *Bartonella* sp. in Different Populations of Fleas. *Curr Microbiol*. 2016 Nov;73(5):704–13.

60. Sevestre, J.; Diarra, A.Z.; Laroche, M.; Almeras, L.; Parola, P. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: an emerging tool for studying the vectors of human infectious diseases. *Future Microbiol*. 2021 Mar;16:323–40.

61. Huynh, L.N.; Diarra, A.Z.; Pham, Q.L.; Le-Viet, N.; Berenger, J.M.; Ho, V.H.; *et al*. Morphological, molecular and MALDI-TOF MS identification of ticks and tick-associated pathogens in Vietnam. *PLoS Negl Trop Dis*. 2021 Sep;15(9):e0009813.

62. Tahir, D.; Almeras, L.; Varloud, M.; Raoult, D.; Davoust, B.; Parola, P. Assessment of MALDI-TOF mass spectrometry for filariae detection in *Aedes aegypti* mosquitoes. *PLoS Negl Trop Dis*. 2017 Dec 20;11(12):e0006093

63. Diarra, A.Z.; Almeras, L; Laroche, M.; Berenger, J.M.; Koné, A.K.; Bocoum, Z.; *et al*. Molecular and MALDI-TOF identification of ticks and tick-associated bacteria in Mali. *PLoS Negl Trop Dis*. 2017 Jul;11(7):e0005762.

64. Werren, J.H.; Baldo, L.; Clark, M.E. *Wolbachia*: master manipulators of invertebrate biology. *Nat Rev Microbiol*. 2008 Oct;6(10):741–51.

65. Tay, S.T. *Wolbachia* endosymbionts, *Rickettsia felis* and *Bartonella* species, in *Ctenocephalides felis* fleas in a tropical region. *Journal of Vector Ecology*. 2013 Jun;38(1):200–2.

66. Gonçalves, .LR.; Harrus, S.; Herrera, H.M.; Gutiérrez, R.; Pedrassani, D.; Nantes, W.A.G.; *et al*. Low occurrence of *Bartonella* in synanthropic mammals and associated ectoparasites in peri-urban areas from Central-Western and Southern Brazil. *Acta Tropica*. 2020 Jul;207:105513.

67. Parola, P.; Raoult, D. Tropical rickettsioses. *Clinics in Dermatology*. 2006 May 1;24(3):191–200.

68. Parola, P; Shpynov, S.; Montoya, M.; Lopez, M.; Houpikian, P.; Zeaiter, Z.; *et al*. First molecular evidence of new *Bartonella* spp. in fleas and a tick from Peru. *Am J Trop Med Hyg*. 2002 Aug;67(2):135–6.

69. Diarra, A.Z; Kone, A.K.; Doumbo, N.S.; Laroche, M.; Diatta, G.; Atteynin,e S.A.; *et al*. Molecular Detection of Microorganisms Associated with Small Mammals and Their Ectoparasites in Mali. *Am J Trop Med Hyg*. 2020 Dec;103(6):2542–51.

70. Yin, X.; Zhao, S.; Yan, B; Tian, Y.; Ba, T.; Zhang, J.; *et al*. *Bartonella rochalimae*, *B. grahamii*, *B. elizabethae*, and *Wolbachia* spp. in Fleas from Wild Rodents near the China-Kazakhstan Border. *Korean J Parasitol*. 2019 Oct;57(5):553–9.

71. Gundi, V.A.K.B.; Taylor, C.; Raoult, D.; La Scola, B. *Bartonella rattaustraliani* sp. nov., *Bartonella queenslandensis* sp. nov. and *Bartonella coopersplainsensis* sp. nov., identified in Australian rats. *International Journal Of Systematic And Evolutionary Microbiology*. 2009 Dec 1;59(12):2956–61.

72. Saengsawang, P.; Kaewmongkol, G.; Phoosangwalthong, P.; Chimnoi, W.; Inpankaew, T. Detection of zoonotic *Bartonella* species in ticks and fleas parasitizing free-ranging cats and dogs residing in temples of Bangkok, Thailand. *Veterinary Parasitology*: Regional Studies and Reports. 2021 Jul;25:100612.

73. Inoue, K.; Maruyama, S.; Kabeya, H.; Yamada, N.; Ohashi, N.; Sato ,Y.; *et al*. Prevalence and genetic diversity of *Bartonella* species isolated from wild rodents in Japan. *Appl Environ Microbiol*. 2008 Aug;74(16):5086–92.

74. Gundi, V.A.K.B.; Kosoy, M.Y.; Myint, K.S.A.; Shrestha, S.K.; Shrestha, M.P.; Pavlin, J.A.; *et al*. Prevalence and genetic diversity of bartonella species detected in different tissues of small mammals in Nepal. *Appl Environ Microbiol*. 2010 Dec;76(24):8247–54.

75. Winoto, I.L.; Goethert, H.; Ibrahim, I.N.; Yuniherlina, I.; Stoops, C.; Susanti, I.; *et al*. *Bartonella* species in rodents and shrews in the greater Jakarta area. *Southeast Asian J Trop Med Public Health*. 2005 Nov;36(6):1523–9.

76. Jiyipong. T.; Morand, S.; Jittapalapong, S.; Rolain, J.M. *Bartonella* spp. infections in rodents of Cambodia, Lao PDR, and Thailand: identifying risky habitats. *Vector Borne Zoonotic Dis*. 2015 Jan;15(1):48–55.

77. Logan, J.M.J.; Hall, J.L; Chalker, V.J.; O'Connell, B.; Birtles, R.J. *Bartonella clarridgeiae* infection in a patient with aortic root abscess and endocarditis. *Access Microbiol*. 2019 Oct 10;1(10):e000064.

78. Kordick, D.L; Hilyard, E.J.; Hadfield, T.L.; Wilson, K.H.; Steigerwalt, A.G.; Brenner, D.J.; *et al*. *Bartonella clarridgeiae*, a newly recognized zoonotic pathogen causing inoculation papules, fever, and lymphadenopathy (cat scratch disease). *J Clin Microbiol*. 1997 Jul;35(7):1813–8.

79. André, M.R.; Canola, R.A.M.; Braz, J.B.; Perossi, I.F.S.; Calchi ,A.C.; Ikeda, P.; *et al*. Aortic valve endocarditis due to *Bartonella clarridgeiae* in a dog in Brazil. *Rev Bras Parasitol Vet*. 2019 Dec;28(4):661–70.

80. Loan, H.K.; Cuong, N.V.; Takhampunya, R.; Klangthong, K.; Osikowicz, L.; Kiet, B.T; *et al*. *Bartonella* species and trombiculid mites of rats from the Mekong Delta of Vietnam. *Vector Borne Zoonotic Dis*. 2015 Jan;15(1):40–7.

81. Anh, P..H.; Van, C.N.; Son, N.T.; Tue, N.T.; Kosoy, M.; Woolhouse, M.E.J.; *et al*. Diversity of *Bartonella* spp. in Bats, Southern Vietnam. *Emerg Infect Dis*. 2015 Jul;21(7):1266–7.

82. Parola, P.; Miller, R.S.; McDaniel, P.; Telford, S.R; Rolain, J.M.; Wongsrichanalai, C.; *et al*. Emerging rickettsioses of the Thai-Myanmar border. *Emerg Infect Dis*. 2003 May;9(5):592–5.

83. Chomel, B.B.; Carlos, E.T.; Kasten, R.W.; Yamamoto, K.; Chang, C.C.; Carlos, R.S.; *et al*. *Bartonella henselae* and *Bartonella clarridgeiae* infection in domestic cats from The Philippines. *Am J Trop Med Hyg*. 1999 Apr;60(4):593–7.

84. Marston, E.L; Finkel, B; Regnery, R.L.; Winoto, I.L; Graham, R.R; Wignal, S.; *et al*. Prevalence of *Bartonella henselae* and *Bartonella clarridgeiae* in an urban Indonesian cat population. *Clin Diagn Lab Immunol*. 1999 Jan;6(1):41–4.

85. Mizukami, M.; Sato, S.; Nabeshima, K.; Kabeya, H.; Ueda, D.; Suzuki, K.; *et al*. Molecular survey of *Bartonella rochalimae* in Japanese raccoon dogs (Nyctereutes procyonoides viverrinus). *Journal of Wildlife Diseases*. 2020 Jul 2;56(3):560.

86. Eremeeva, M.E.; Gerns, H.L.; Lydy, S.L.; Goo, J.S.; Ryan, E.T.; Mathew, S.S.; *et al*. Bacteremia, fever, and splenomegaly caused by a newly recognized bartonella species. *N Engl J Med*. 2007 Jun 7;356(23):2381–7.

87. Henn, J.B.; Gabriel, M.W.; Kasten, R.W.; Brown, R.N.; Koehler, J.E.; MacDonald, K.A.; *et al*. Infective Endocarditis in a Dog and the Phylogenetic Relationship of the Associated "*Bartonella rochalimae*" Strain with Isolates from Dogs, Gray Foxes, and a Human. *J Clin Microbiol*. 2009 Mar;47(3):787–90.

88. Saisongkorh, W.; Suputtamongkol, Y.; Raoult, D. Emerging Bartonella in humans and animals in Asia and Australia. *Journal of the Medical Association of Thailand*. 2014;26.

89. Tay, S.T.; Mokhtar, A.S.; Zain, S.N.M.; Low, K.C. Isolation and Molecular Identification of Bartonellae from Wild Rats (Rattus Species) in Malaysia. *Am J Trop Med Hyg*. 2014 Jun 4;90(6):1039–42.

90. Helan, J.N.V.G.; Grinberg, A.; Gedye, K.; Potter, M.; Harrus, S. Molecular detection of *Bartonella coopersplainsensis* and *B. henselae* in rats from New Zealand. *New Zealand Veterinary Journal*. 2018 Sep 3;66(5):257–60.

91. Li, D.M.; Hou, Y.; Song, X.P.; Fu, Y.Q.; Li, G.C.; Li, M.; *et al*. High prevalence and genetic heterogeneity of rodent-borne *Bartonella* species on Heixiazi Island, China. *Appl Environ Microbiol*. 2015 Dec;81(23):7981–92.

92. Mardosaitė-Busaitienė, D.; Radzijevskaja, J.; Balčiauskas, L.; Bratchikov, M.; Jurgelevičius, V.; Paulauskas, A. Prevalence and diversity of *Bartonella* species in small rodents from coastal and continental areas. *Sci Rep*. 2019 Aug 26;9(1):12349.

ARTICLE IV: LITERATURE REVIEW

Mosquitoes and mosquito-borne diseases in Vietnam

Ly Na Huynh, Long Bien Tran, Hong Sang Nguyen, Van Hoang Ho, Philippe Parola, Xuan

Quang Nguyen*

Abstract

Mosquito-borne diseases pose a significant threat to humans in almost every part of the world. Key factors such as global warming, climatic conditions, rapid urbanisation, frequent human relocation, and widespread deforestation significantly increase the number of mosquitoes and mosquito-borne diseases in Vietnam, and elsewhere around the world. In southeast Asia, and notably in Vietnam, national mosquito control programmes contribute to reducing the risk of mosquito-borne disease transmission, including social-economic improvement, early diagnosis, prompt and effective treatment, widespread distribution of insecticide-treated bed nets, and indoor residual chemical spraying in households, change of behaviour, public awareness, and environmental management, however, malaria and dengue remain a threat to public health, while there are no potential vaccines available for most mosquito-borne diseases. The aim of our review is to provide a complete checklist of all Vietnamese mosquitoes that have been recognised, as well as an overview of mosquito-borne diseases in Vietnam to entomological researchers for future surveys of Vietnamese mosquitoes and to decision-makers responsible for vector control tactics. A total of 281 mosquito species of 42 subgenera and 22 genera exist in Vietnam. However, the checklist of Vietnamese mosquitoes is far from being complete as long as further investigations on mosquitoes are carried out. Hence, for effective mosquito control, their accurate identification is a crucial step in differentiating mosquito vectors from non-vectors. Nonetheless, there are several drawbacks to these traditional methods (morphological and molecular identification) such as the need for time-consuming, exorbitantly priced, standard taxonomic keys, expert entomology knowledge to classify species belonging to sibling and cryptic groups, and primer-specific targeting for certain species. MALDI-TOF MS is an easy-to-use, time-saving, and affordable method compared to earlier methods, and a device may be purchased for medical microbiology and then used in

entomology. Its operation is based on the identification of acidic extraction of proteins from an organ of interest. Major mosquito-borne diseases in high-incidence areas of Vietnam include malaria, dengue, and Japanese encephalitis

Review

Mosquitoes and mosquito-borne diseases in Vietnam

Ly Na Huynh[1,2,3], Long Bien Tran[3], Hong Sang Nguyen[3], Van Hoang Ho[3], Philippe Parola[1,2], Xuan Quang Nguyen[3]*

[1] Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France; lynaimpe@gmail.com (LNH); adama-zandiarra@gmail.com (A.Z.D)

[2] IHU-Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13005 Marseille, France.

[3] Institute of Malariology, Parasitology and Entomology, Quy Nhon (IMPE-QN), Vietnam; long-bien19842011@gmail.com (L.B.T); sangimpeqn@gmail.com (H.S.N); ho_hoang64@yahoo.com (V.H.H); philippe.parola@univ-amu.fr (P.P)

* Correspondence: Dr. Xuan Quang Nguyen. Institute of Malariology, Parasitology and Entomology, Quy Nhon (IMPE-QN), MoH Vietnam, Zone 8, Nhon Phu ward, 590000 Quy Nhon city, Binh Dinh province, Vietnam. Phone: +84 (0) 9 13 43 85 57. Email: nxquang134@gmail.com

Simple Summary: Vietnam is one of the tropical countries of Asia where individuals are at high risk of attaining mosquito-borne diseases. Due to rapid urbanization in Vietnam, most of the major cities have immense population growth, along with inadequate control measures over mosquitoes. These factors contributed to a sudden increase in the population of disease vectors that lead to cyclical epidemics of mosquito-borne diseases. This review paper aims to (i) provide a complete checklist of Vietnamese mosquitoes, (ii) provide an overview of mosquito-borne diseases in Vietnam, and (iii) preventive measures for mosquitoes in Vietnam. We list 281 mosquito species, belonging to 42 subgenera of 22 genera. We found that three genera, namely, *Anopheles*, *Aedes*, and *Culex* are found to be potential vectors for mosquito-borne diseases in Vietnam. We found dengue and malaria are the most common mosquito-borne diseases in Vietnam with about 320,702 cases and 54 deaths in the 2019 outbreak and 4548 clinical cases and six deaths, respectively. We suggest that mosquito-borne diseases could be effectively controlled and prevented through mechanical, chemical, biological, and genetic methods.

Abstract: Mosquito-borne diseases pose a significant threat to humans in almost every part of the world. Key factors such as global warming, climatic conditions, rapid urbanisation, frequent human relocation, and widespread deforestation significantly increase the number of mosquitoes and mosquito-borne diseases in Vietnam, and elsewhere around the world. In southeast Asia, and notably in Vietnam, national mosquito control programmes contribute to reducing the risk of mosquito-borne disease transmission, however, malaria and dengue remain a threat to public health. The aim of our review is to provide a complete checklist of all Vietnamese mosquitoes that have been recognised, as well as an overview of mosquito-borne diseases in Vietnam. A total of 281 mosquito species of 42 subgenera and 22 genera exist in Vietnam. Of those, *Anopheles*, *Aedes*, and *Culex* are found to be potential vectors for mosquito-borne diseases. Major mosquito-borne diseases in high-incidence areas of Vietnam include malaria, dengue, and Japanese encephalitis. This review may be useful to entomological researchers for future surveys of Vietnamese mosquitoes and to decision-makers responsible for vector control tactics.

1. Introduction

Mosquitoes are vectors of disease and can transmit many infectious pathogens (arboviruses, filariae, and protozoans) that cause common and emerging diseases, specifically malaria, dengue, Zika, chikungunya, and Japanese encephalitis, between humans or from other creatures to humans [1]. Mosquito-borne diseases are widely distributed in almost all countries of the world, but are mainly concentrated in tropical and subtropical regions of the globe with warm and humid climates [2]. There are 3,563 valid species, belonging to three genera (*Anopheles*, *Aedes*, and *Culex*) within two subfamilies (Anophelinae and Culicinae) [3,4]. The mosquito vectors of the *Anopheles* genus cause malaria and filariasis, while the *Aedes* genus causes dengue virus (DENV), chikungunya virus (CHIKV), Zika virus (ZIKV), yellow fever virus (YFV), and the *Culex* genus cause filariasis, Japanese encephalitis virus (JEV) and West Nile virus (WNV) [5]. According to the World Health Organization (WHO, 2020), every year about 219 million malaria cases and 96 million symptomatic dengue cases, leading to more than 445,000 and 40,000 deaths, respectively, are recorded globally [4,6]. There are no potential vaccines available for most mosquito-borne diseases. The WHO has, therefore, declared that the key preventive measures for controlling mosquito-borne diseases are a change of behaviour, public awareness, and environmental management [6].

Vietnam is a tropical country in Asia where individuals are at high risk of acquiring mosquito-borne diseases such as malaria, dengue, Zika, Japanese encephalitis, and lymphatic filariasis [7]. Although malaria was previously endemic in many parts of Vietnam, it has recently been significantly reduced following vector control efforts by the Vietnamese government. These measures included prompt case detection and treatment with pyronaridine-artesunate (Pyramax) which is used for diversifying anti-malarial therapy in artemisinin- and piperaquine-resistant *Plasmodium falciparum* parasite regions [8], the widespread distribution of insecticide-treated bed nets (ITNs), long-lasting insecticide-treated bed nets/long-lasting insecticide-treated hammock nets (LLTNs/LLHNs), and the regular the use of pyrethroids through indoor residual spraying (IRS) in households [9–14]. However, malaria continues to persist as an important public health threat in the Central Highlands and the southern forested and mountainous regions [15,16], where almost all ethnic minority groups live in impoverished socio-economic conditions. It mainly affects people such as farmers and/or forest-goers, as well as temporary migrants moving from endemic regions [17]. In addition, vector resistance to chemical insecticide, has been found in every main species. *Anopheles minimus* sensu lato (s.l.) was found to be pyrethroid-resistant in northern Vietnam, *Anopheles dirus* s.l. showed possible tolerance to type II pyrethroids in central Vietnam, and *Anopheles epiroticus* showed high resistance to pyrethroids in the Mekong Delta region [18,19]. The drug-resistant malaria strains in particular pose a major potential threat to extensive malaria control and elimination strategies planned for the whole country before 2030 [12,20,21]. Cases of the multidrug resistance of the *P. falciparum* malaria parasite in Vietnam and other southeast Asian countries such as Laos, Cambodia, Thailand, and Myanmar, have been published [15,22,23]. Dengue fever (DF) is the fastest spreading mosquito-borne arboviral infection, which is transmitted to humans through the bites of infected female *Aedes aegypti* or *Aedes albopictus* mosquitoes [24]. Vietnam has been ranked among the five countries with the heaviest dengue burden in the Asian-Pacific region [25,26]. Due to rapid urbanisation, most of the major cities in Vietnam have experienced immense population growth, coupled with inadequate mosquito control measures. Additionally, the country faces the risk of climate change [27] and extreme weather variability, with widespread flooding due to rising sea levels. Other unexpected outcomes of climate change include rising temperatures and decreasing rainfall. These factors have contributed to a sudden increase in the population of disease vectors that lead to cyclical epidemics of mosquito-borne diseases [28].

This is the first review that provides a complete checklist of Vietnamese mosquitoes along with a comprehensive and systematic overview of mosquito-borne diseases in Vietnam. We hope it may be useful to decision-makers responsible for vector control strategies and to researchers for future surveys on mosquitoes.

2. Mosquitoes and medical importance in Vietnam

In Vietnam, detailed studies on mosquitoes (particularly on the *Anopheles*, *Culex*, and *Aedes* species) were initiated after the first outbreak of the dengue virus in 1959 [28]. However, the first detailed report on mosquitoes and their relationship to human diseases in Vietnam was proposed by Parrish in 1968 [29]. In this report, Parrish identified 94 different mosquito species from ten United States Air Force (USAF) installations in Vietnam between June 1966 and June 1968. Of these, 22 species were reported as main vectors of human diseases [29]. In 1970, Reinert [30] and Tyson [31] improved the catalogue of *Culex* and *Aedes* mosquitoes in SEA countries including Vietnam, Malaysia, Indonesia, and the Philippines.

In 1974, Thoa [32] collected and examined around 107,000 mosquitoes in and around poultry houses and piggeries in Saigon city (now known as Ho Chi Minh City) between 1972–1973 and noted that the *Culex* species (particularly *Culex tritaeniorhynchus*) was predominant. Later, Thuan [33] studied and reported the evolution of *Anopheles* mosquitoes in the Da Nang, Quang Nam, Phu Khanh, Nghia Binh, and Thuan Hai provinces, between 1975 and 1986. Since then, multiple studies on the diversity of medical mosquito vectors in Vietnam have been published [34]. Bui *et al.* (2008) listed 191 species and subspecies, 28 subgenera and 35 genera, and the authors compared their research with those published from US military sources during the Vietnam war. However, these data were not exhaustive, as they did not update all the reported research by domestic authors [35]. More recently, Nguyen (2015) listed 255 species in 21 genera and 42 subgenera. The author's list was based on research papers published by domestic and foreign researchers [36]. Nevertheless, none of them are documented as a literature review of existing mosquito species, along with their associated mosquito-borne diseases. Hence, in this review, we provide a complete checklist of all recognised mosquitoes in Vietnam. It was compiled from multiple reliable studies published both nationally and internationally by Parrish (1969) and Grothaus *et al.* (1971) [29,30], Reinert (1973) [37], Vu (1984) [34], Harbach (2007) [38], Bui *et al.* (2008) [35] and Nguyen (2015) [36].

Our research reported the existence of a total of 281 Vietnamese mosquito species in 42 subgenera and 22 genera, of which the main vectors were 66 *Anopheles*, 51 *Aedes*, 50 *Culex*, one *Aedeomyia*, 12 *Armigeres*, three *Coquillettidia*, one *Ficalbia*, seven *Heizmannia*, one *Hodgesia*, one *Kimia*, four *Lutzia*, three *Malaya*, nine *Mansonia*, three *Mimomyia*, 24 *Ochlerotatus*, three *Orthopodomyia*, three *Topomyia*, four *Toxorhynchites*, six *Tripteroides*, one *Udaya*, 20 *Uranotaenia*, and eight *Verrallina* species [36,39]. These findings are presented in Tables 1 and 2. The distribution of the primary mosquito vectors in Vietnam and breeding and resting sites are shown in Figs. 1A and 1B

Figure 1. Distribution map of the major vectors [**A**]. Landscapes of larval breeding and adult resting habitats of *Aedes aegypti*, *Aedes albopictus*, *Culex quinquefasciatus* [**B**]

3. Mosquito-associated microorganims and human diseases

Malaria

Malaria is a protozoal infection of erythrocytes and ranks fourth among parasitic diseases affecting birds, reptiles, humans, and other mammals [4,5] Human malaria is also known as ague, marsh fever, paludism, and intermittent fever [4]. Of the 172 known *Plasmodium* species, only four, namely *P. falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*, are commonly known to infect humans with malaria [5]. *P. falciparum* has caused the greatest morbidity and mortality to date, with several hundreds of millions of clinical malaria cases [40].

In Vietnam, malaria became a serious public health problem in the late 1980s and early 1990s [41]. In 1991, the National Malaria Control Programme (NMCP), based on ITNs, IRS, and early diagnosis and treatment of malaria (EDTM), was approved. Since then, there has been a reduction in malaria cases between 1991, when there were estimated to be 1,674,000 uncomplicated and severe clinical cases and 4,650 deaths, and 2017, when there were estimated to be 4,548 clinical cases and six deaths [41–43]. Nevertheless, as is the case in many countries in the Asia-Pacific region, Vietnam faces the challenges of rapidly evolving multi-drug resistant parasites [44], changes in the outdoor feeding and early biting and resting behaviour of some mosquitoes, as well as insecticide-resistant mosquitoes, which impact the malarial control and elimination strategies which were expected to eradicate the disease by 2030.

Early in the 19th century, Leger (1910) published 15 species of *Anopheles* mosquitoes in Vietnam. Gallilard and Dang (1946) built the taxonomic key of 22 *Anopheles* mosquito species. Tran (1995), published a list of 55 species nationwide [45]. To date, 64 *Anopheles* species have been identified, but, only 15 *Anopheles* species have formally been recognised as likely to transmit malaria [46].

149

Table 1. Checklist of mosquito species reported in Vietnam

Genus	Species				
	Before 1970 [34–36]		**1970-present** [30–33,37]		
	An. aconitus	*An. philippinensis*	*An. aberrans*	*An. harrisoni*	*An. paraliae*
	An. annularis	*An. sinensis*	*An. alongensis*	*An. indefinitus*	*An. pseudojamesi*
	An. argyropus	*An. splendidus*	*An. asiaticus*	*An. insulaeflorum*	*An. pseudowillmori*
	An. barbirostris	*An. subpictus*	*An. baezai*	*An. interruptus*	*An. pursati*
	An. campestris	*An. tessellatus*	*An. baileyi*	*An. jamesii*	*An. rampae*
	An. crawfordi	*An.umbrosus*	*An. barbumbrosus*	*An. kochi*	*An. sawadwongporni*
	An. introlatus	*An. vagus*	*An. bengalensis*	*An. letifer*	*An. separatus*
Anopheles	*An. jeyporiensis*	*An. varuna*	*An. cucphuongensi*	*An. lindesayi*	*An. sintonoides*
	An. karwari		*An. culicifacies*	*An. monstrosus*	*An. stephensi*
	An. lesteri		*An. dangi*	*An. nimpe*	*An. takasagoensis*
	An. maculatus		*An. dirus*	*An. nitidus*	*An. vietnamensis*
	An. minimus		*An. donaldi*	*An. nivipes*	*An. whartoni*
	An. nigerrimus		*An. dravidicus*	*An. notanandai*	*An. willmori*
	An. pallidus		*An. epiropticus*	*An. palmatus*	
	An. peditaeniatus		*An. gigas*	*An. pampanai*	
	Ae. aegypti	*Ae. mediolineatus*	*Ae. alongi*	*Ae. helenae*	*Ae. novonivveus*
	Ae. albolineatus	*Ae. nivens*	*Ae. annandalei*	*Ae. hirsutipleura*	*Ae. pampagensis*
	Ae. albopictus	*Ae. niveoscutellum*	*Ae. andamanensis*	*Ae. gardnerii imitator*	*Ae. patriciae*
	Ae. alboscutellatus	*Ae. ostentatio*	*Ae. agrestis*	*Ae. ibis*	*Ae. poecilus*
	Ae. amesii	*Ae. pseudalbopictus*	*Ae. caecus*	*Ae. jamesii*	*Ae. prominens*
Aedes	*Ae. assamensis*	*Ae. taeniorhynchoites*	*Ae. cancricomes*	*Ae. macfarlanei*	*Ae. saxicola*
	Ae. aureostriatus	*Ae. tonkinensils*	*Ae. chrysolineatus*	*Ae. malayensis*	*Ae. thailandensis*
	Ae. dux	*Ae. vallistris*	*Ae. culicinus*	*Ae. manhi*	*Ae. uniformis*
	Ae. imprimens	*Ae. vexans*	*Ae. desmotes*	*Ae. mediopunctatus*	*Ae. vittatus*
	Ae. laniger		*Ae. eldridgei*	*Ae. niveoides*	*Ae. w-albus*
	Ae. lineatopennis		*Ae. elsiae*		
	Cx. annulus	*Cx. peytoni*	*Cx. alienus*	*Cx. malayi*	
	Cx. bitaeniorhynchus	*Cx. pseudosinensis*	*Cx. alis*	*Cx. mimulus*	
	Cx. brevipalpis	*Cx. pseudovishnui*	*Cx. bernardi*	*Cx. minutissimus*	
	Cx. fuscanus	*Cx. quadripalpis*	*Cx. bicornutus*	*Cx. murrelli*	
	Cx. fuscocephala	*Cx. quinquefasciatus*	*Cx. cinctellus*	*Cx. pallidothorax*	
Culex	*Cx. fuscocephalus*	*Cx. raptor*	*Cx. curtipalpis*	*Cx. scanloni*	
	Cx. gelidus	*Cx. reidi*	*Cx. edwardsi*	*Cx. sumatranus*	
	Cx. incomptus	*Cx. rubithoracis*	*Cx. foliatus*	*Cx. variatus*	
	Cx. khazani	*Cx. sinensis*	*Cx. fragilis*	*Cx. viridiventer*	
	Cx. mimeticus	*Cx. sitiens*	*Cx. hutchinsoni*	*Cx. vishnui*	
	Cx. minor	*Cx. tritaeniorhynchus*	*Cx. infantulus*	*Cx. wilfredi*	
	Cx. nigropunctatus	*Cx. whitei*	*Cx. infula*		

| *Cx. pholster*	*Cx. whitmorei*	*Cx. macdonaldi*		

Three vectors have been incriminated as playing a role of primary vector in malaria transmission, namely *An. dirus* Peyton & Harrison, *An. minimus* Theobald, and *An. epiroticus* Linton & Harbach (Sundaicus complex) [18,47–49]. Other secondary vectors are *Anopheles aconitus* Dönitz, *Anopheles campestris* Linnaeus, *Anopheles culicifacies* Giles, *Anopheles indefinitus* Ludlow, *Anopheles interruptus* Puri, *Anopheles jeyporiensis* James, *Anopheles maculatus* Theobald, *Anopheles lesteri* Baisas & Hu, *Anopheles nimpe* Nguyen, Tran & Harbach, *Anopheles sinensis* Wiedemann, *Anopheles subpictus* Grassi, and *Anopheles vagus* Dönitz, which may be considered to contribute towards the transmission of malaria outside of forested regions [10].

(**a**) (**b**) (**c**)
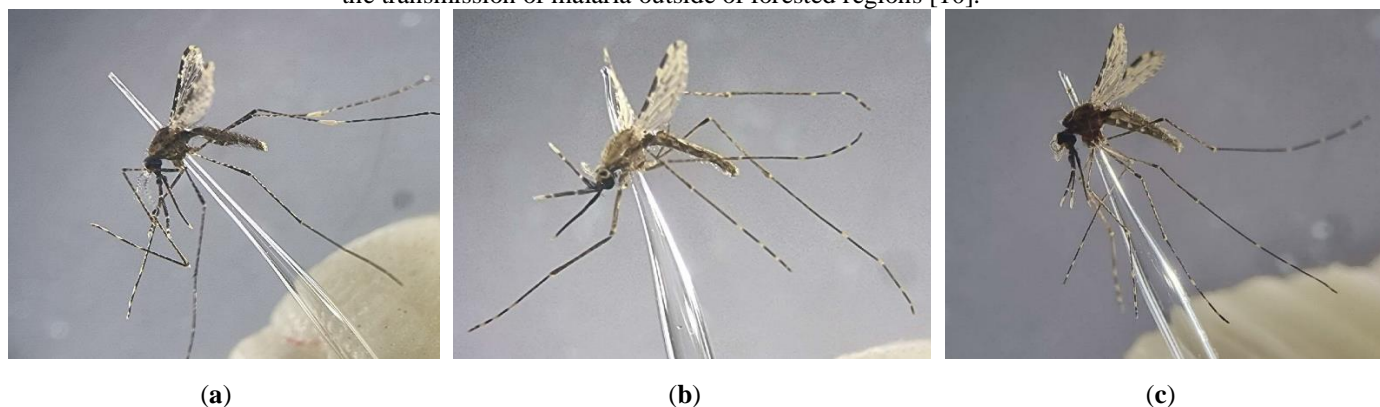
Figure 2. Photographs of three mosquito species which are known to be major malaria vectors in Vietnam: (**a**) *Anopheles dirus*; (**b**) *Anopheles minimus*; (**c**) *Anopheles epiroticus*.

Anopheles dirus sensu stricto (s.s.) Peyton & Harrison (Fig. 2a), 1979, spread to forested and mountain areas from central and south-eastern region of Vietnam [50]. *An. dirus* s.s. belongs to the *Anopheles* (*Cellia*) *leucosphyrus* group, with 20 member species [51]. This species, previously known as *Anopheles balabacensis*, comes from Vietnam and other SEA countries [43]. Recently, it was formally named *An. dirus* s.s. (species A) [51]. Of the four *Plasmodium* species reported in Vietnam and known to be true parasites of humans, *P. falciparum* (64%) and *P. vivax* (35%) [52] have commonly been found, while *P. ovale*, *P. malariae* are also present but at low levels [52–54]. In 2009, a fifth parasite species was discovered, *Plasmodium knowlesi*, a macaque parasite that was recognised for the first time in a case of human malaria in a nine-year-old child in Khanh Phu in the Khanh Hoa province of Vietnam [55]. Interestingly, *An. dirus* was incriminated as the vector for carrying sporozoites of *P. knowlesi*, *P. falciparum*, and *P. vivax*, in a paper published by Charmand [56]. *An. minimus* s.l. (Fig. 2b) belongs to the Minimus subgroup. The *An. minimus* complex comprises three species, of which two are known to occur in the Greater Mekong Subregion (GMS), namely *An. minimus* Theobald (formerly *An. minimus* A), and *Anopheles harrisoni* Harbach and Manguin (formerly *An. minimus* C) [18]. *An. minimus* s.l. is widespread in hilly and forested areas nationwide. These mosquitoes breed in small, slow-flowing streams with aquatic vegetation, and clear water in full sunlight [57]. *An. epiroticus* (formerly *Anopheles sundaicus* species A) (Fig. 2c), which is distinguished by brackish water populations in sunlight, only occurs in coastal areas of southern Vietnam [58].

Dengue

Dengue or dengue fever is the most prominent mosquito-borne virus, leading to approximately 100 million symptomatic cases in about 100 tropical-zone countries every year. According to the WHO (2021), the number of dengue cases increased more than eight-fold from 505,430 cases in 2000 to 5.2 million cases in 2019, with an increase in the death rate from 960 in 2000 to 4032 in 2015 [59]. It is estimated that approximately 70% of these cases were in Asia, with the most affected countries being, in order, the Philippines (420,000), Vietnam (320,000), Malaysia (131,000), and Bangladesh (101,000) [59]. The dengue virus (family *Flaviviridae*) has five distinct serotypes (DENV-1 to

186   DENV-5) and serotypes 1-4 are widely spread globally [60]. Infection with any of these five sero-
187   types can cause illnesses such as dengue fever and dengue shock syndrome [59]. Generally, the
188   dengue virus is transmitted in humans through bites of the female *Aedes* mosquito.

189   **Table 2.** Checklist of other mosquito species reported in Vietnam

Genus	**Species**		
	Before 1970 [31,35]	**1970-present** [30–33]	
Aedeomyia	*Ad. Catasticta*	-	
Armigeres	*Arm. flavus*	*Arm. annulitarsis*	*Arm. durhami*	*Arm. moultoni*
	Arm. subalbatus	*Arm. aureolineatus*	*Arm. kuchingensis*	*Arm. pectinatus*
		Arm. cingulatus	*Arm. longipalpis*	
		Arm. dolichocephalus	*Arm. magnus*	
Coquillettidia	-	*Cq. crassipes*	*Cq. ochracea*	*Cq. nigrosignata*
Ficalbia	*Fi. minima*	-	
Heizmannia	-	*Hz. communis*	*Hz. greenii*	*Hz. scintillans*
		Hz. complex	*Hz. persimilis*	
		Hz. covelli	*Hz. reidi*	
Hodgesia	*Ho. malayi*	-	
Kimia	-	*Km. decorabilis*	
Lutzia	-*Lt. raptor*	*Lt. fuscanus*	*Lt. halifaxii*	*Lt. vorax*
Malaya	*Ml. jacobsoni*	*Ml. genurostris*	*Malaya* sp.	
Mansonia	*Ma. annulifera*	*Ma. annulata*		
	Ma. crassipes	*Ma. bonneae*		
	Ma. nigrosignata	*Ma. dives*		
	Ma. ochracea	*Ma. indiana*		
	Ma. uniformis			
Mimomyia	-	*Mi. chamberlaini metallica*	*Mi. hybrida*	*Mi. luzonensis*
Ochlerotatus		*Och. assamensis*	*Och. macfarlanei*	*Och. saxicola*
		Och. aureostriatus	*Och. mikrokopion*	*Och. scatophgoides*
		Och. chrysolineatus	*Och. niveoides*	*Och. tonkinensis*
		Och. dissimilis	*Och. niveus*	*Och. vigilax*
		Och. elsiae	*Och. novoniveus*	*Ochlerotatus* sp.1
		Och. khazani	*Och. poicilius*	*Ochlerotatus* sp.2
		Och. laniger	*Och. prominens*	*Ochlerotatus* sp.3
		Och. longirostris	*Och. pseudotaeniatus*	*Ochlerotatus* sp.4
Orthopodomyia	-	*Or. albipes*	*Or. andamanensis*	*Or. anopheloides*
Topomyia	-	*To. Gracilis*	*Topomyia* sp.1	*Topomyia* sp.2
Toxorhynchites	*Tx. splendens*	*Tx. albipes*	*Tx. Kempi*	*Toxorhynchites* sp.
Tripteroides	*Tp. aranoides*	*Tp. powelli*	*Tp. similis*	*Tp. tenax*
		Tp. proximus	*Tp. tarsalis*	
Udaya	-	*Ud. argyrurus*		
Uranotaenia	*Ura. annandalei*	*Ura. bicolor*	*Ura. koli*	*Ura. spiculosa*

	Ura. campestris	*Ura. bimaculata*	*Ura. lateralis*	*Uranotaenia* sp.
	Ura. macfarlanei	*Ura. bimaculiala*	*Ura. longirostris*	
	Ura. maxima	*Ura. demeilloni*	*Ura. lutescens*	
	Ura. obscura	*Ura. edwardsi*	*Ura. nivipleura*	
	Ura. recondita	*Ura. hongayi*	*Ura. rampae*	
Verrallina	-	*Ver. andamanensis*	*Ver. consonensis*	*Ver. unca*
		Ver. butleri	*Ver. dux*	*Ver. vallistris*
		Ver. clavata	*Ver. nigrotarsis*	

Vietnam is the country that is second most affected by the dengue virus in Asia, as well as in the world, and the first dengue case was reported in 1959 [7,59,61]. However, the largest dengue outbreak was reported in 2019, with about 320,702 cases and 54 deaths between January and December [61]. According to a report from the Vietnamese Ministry of Health, a 2.5-fold increase in the number of dengue cases was noted in the 2019 dengue outbreak, compared to cases in 2018 [61]. It has been reported that the dengue virus (mostly serotypes 1-4) has been highly distributed across Vietnam, from the southern region into the central and northern regions of the country. For instance, Nha Trang city in the central region of Vietnam recorded 12,655 dengue fever cases between 2006 and 2016 [62]. The capital city, Hanoi, (northern region) has witnessed many significant dengue outbreaks over the last decade. The largest outbreak was recorded in 2017 with 37,651 cases and seven deaths [7]. The incidence rate of the dengue virus varies for each province and region, and the most affected cases are recorded in the southern part of the country compared to other regions [63,64]. To date, Vietnam has recorded an increase of about 15,000 dengue cases compared to statistics in the past 10 days, raising the total number of cases in 2022 as of July 2022 to 92,000 cases and 36 deaths [65,66]. The main hotspot areas for dengue fever are in southern Vietnam, specifically Ho Chi Minh with 21,750 cases, an increase of more than 181% compared to the same period last year, and leading to nine deaths [65,67], followed by the provinces of Binh Duong (5,000 cases and eight deaths) [68], Dong Nai (3,500 cases and three deaths), and An Giang (4,400 cases) [69]. These provinces have all witnessed an increasing number of infections. Due to the high population growth and rapid urbanisation, the biggest city, Ho Chi Minh, is well-know as critical urban centre for the transmission of dengue virus.

Aedes aegypti (Fig. 3d) and *Ae. albopictus* (Fig. 3e) are two main vectors that cause dengue fever in Vietnam. *Ae. aegypti* is a dominant vector in urban regions of Vietnam, mainly breeding in flower vases, jars, and plastic buckets. *Ae. albopictus* breeds with lower density in both urban and peri-urban areas than *Ae. aegypti*, due to climate change, migration, and urbanisation. The factors influencing the distribution of the dengue virus in Vietnam have been found to be industrialisation, urbanisation, and climate change [70].

Zika virus infection

Zika virus is an arbovirus (*Flavivirus* genus) with a rapid geographic spread [71]. It was first isolated from the blood samples of a rhesus monkey, in the Ziika Forest of Uganda, in 1947, during epidemiological studies of yellow fever [72,73]. However, the first case in humans was isolated in a Nigerian female (10 years old) in 1954 [74] and, outside the African continent, it was first identified in SEA (Malaysia 1966) and Indonesia (1977) [75,76]. However, the largest outbreak in humans was reported on the island of Yap in 2007, where 75% of the population was infected [77,78]. Later, new epidemics broke out in French Polynesia (in late 2013) [79], the South Pacific (during 2014 and 2016), region of the Americas (in 2015), and Brazil (in 2015), leading the WHO to declare "congenital Zika syndrome" as a global public health emergency in February 2016 [76]

Serological screening during these epidemics identified that the *Aedes* species, including *Aedes hensilli* in Yap island, *Aedes polynesiensis* and *Ae. aegypti* in French Polynesia, and *Aedes albopictus* and *Ae. aegypti* in much of the Americas, are the probable vectors of Zika infection [71]. However, *Ae. aegypti* is the leading vector in urban areas, while *Ae. albopictus* occurs in both urban and rural areas [80]. In addition, the Zika virus has also been rarely isolated from *Anopheles, Culex,* and *Mansonia* species [81]. In recent ages, some evidence has been found that the Zika virus can also be transmitted by perinatal transmission, transfusions, and sexually [73]. The general clinical symptoms of Zika virus are arthritis or arthralgia, conjunctivitis, fever, Guillain-Barré syndrome, headache, muscle and joint pain, myalgia, rash, retro-orbital pain, tiredness, and vomiting [82,83].
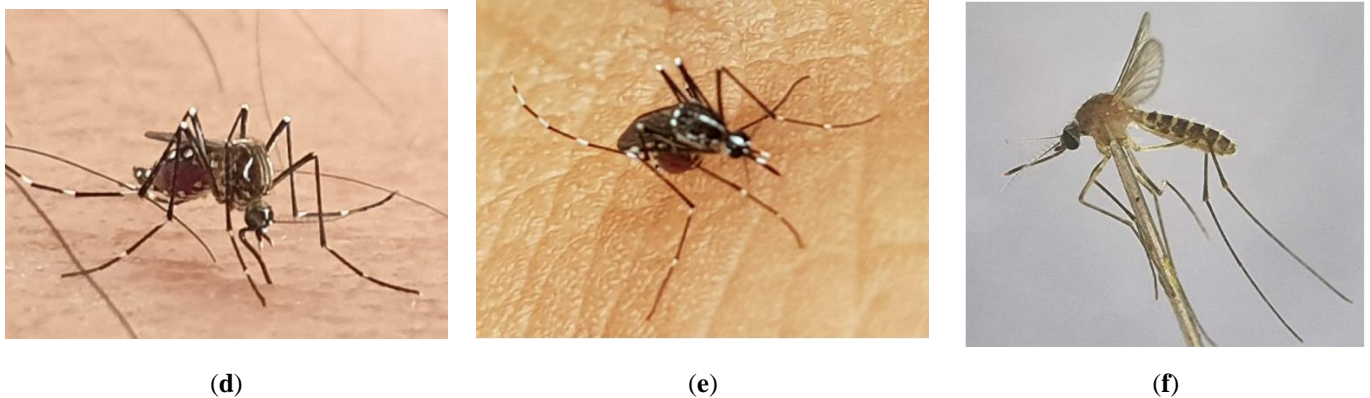

(**d**) (**e**) (**f**)

Figure 3: Photographs of three mosquito species which are potential vectors of several viruses, including dengue, Zika, and Japanese encephalitis in Vietnam: (**d**) *Aedes aegypti*; (**e**) *Aedes albopictus*; (**f**) *Culex quinquefasciatus*.

Vietnam is one of the top five countries that have been recognised as sentinel indicators for Zika virus transmission [84]. In Vietnam, the first Zika infection was identified by Real-Time PCR in two children from the Long An province and Ho Chi Minh city in 2013 [85]. Later, it was identified in two females from Nha Trang city and Ho Chi Minh city in April 2016 [86]. During this first outbreak in 2016, around ten provinces in the Central Highlands and southern regions were strongly affected with 212 cases [86]. Furthermore, the number of cases dropped significantly to 24 cases in 2017, and one case in 2018 86,87]. All cases reported in Vietnam were recognised to be local, vector-borne infections [87]

In Vietnam, the *Aedes* species is the main vector reported to cause transmission of the Zika virus [87]. The entomological report during the 2016 outbreak noted that Zika virus was mainly transmitted by the *Ae. aegypti* vector, which mostly survives in water stored at home [87,88]. In addition, the significant growth of the density of the vector was found to be due to (a) the impact of urbanisation; (b) climate change; (c) lack of awareness on the importance of eliminating the breeding areas of mosquitoes [88].

Japanese encephalitis

Japanese encephalitis is caused by a *Flavivirus*, which is the most common vector-borne disease in Asia and Pacific countries. It affects 67,900 people every year all over the globe [89]. Around 75% of these cases are reported among children 0–14 years old. The general clinical symptoms of viral encephalitis are disorientation, headache, high fever, neck stiffness, seizures, spastic paralysis, and coma [89,90]. Some serological screening studies have reported that the encephalitis virus may cause severe symptoms including abortion and stillbirth in animals such as horses and dogs [91]. Generally, pigs and birds act as reservoirs of the Japanese encephalitis virus, whereas humans and other animals are considered dead-end hosts [91,92]. The enzootic transmission cycle of Japanese encephalitis involves mosquitoes, birds, and pigs. However, there is no evidence that mosquitoes play a significant role in the epidemiology of the encephalitis virus [92].

In the 1930s, the first isolate of the Japanese encephalitis virus was identified from *Culex tritaeniorhynchus* and 30 other mosquito species were later detected [93]. However, mosquitoes from *Culex tritaeniorhynchus*, *Culex gelidus*, *Culex fuscocephala*, *Culex vishnui*, and *Culex quinquefasciatus* (Fig. 3f) are well-recognised vectors and capable of transmitting flavivirus both naturally and experimentally [94,95], while mosquitoes from *Aedes*, *Anopheles*, *Armigeres*, and *Mansonia* genera are proven to transmit it experimentally [93,96,97]. Favourable breeding areas for these mosquito species are found to be rice fields, wastewater, and irrigation systems [92,98,99]. Encephalitis is, therefore, most common in countryside areas.

In Vietnam, the Japanese encephalitis virus circulated in both rural and urban areas. In 1951, the first case of Japanese encephalitis virus was isolated in Vietnam [100]. Until 2003, the encephalitis virus was endemic throughout Vietnam, with 1,000–3,000 cases annually [92,101]. Later, the incidence rate of the encephalitis virus in the Vietnamese population reduced dramatically after the implementation of immunisation and Japanese encephalitis vaccination programmes [100]. In Vietnam, the population of *Culex* species was predominant and its abundance was significantly connected with the density of cattle [102]. *Cx. tritaeniorhynchus*, *Cx. vishnui* s.l., and *Cx. quinquefasciatus* are the major vectors that cause encephalitis in Vietnam [103,104]. Species in the *Cx. vishnui* subgroup, including *Cx. tritaeniorhynchus*, *Cx. vishnui*, and *Cx. pseudovishnui*, breed locally on a large scale in aquatic habitats, i.e., rice areas, puddles, ditches, and cisterns. The most common breeding habitat is the rice-field ecosystem [105]. *Culex quinquefasciatus* is a member of the *Culex pipiens* complex.

Lymphatic filariasis

Lymphatic filariasis (LF) is a rapidly spreading and neglected tropical disease that causes permanent disruption of the human lymphatic system. It is widely distributed in tropical and subtropical countries in Africa, America, Asia, and the western Pacific [106,107]. Globally, the infection of lymphatic filariasis increased significantly from 120 million cases in 1997 to 56 million cases in 2017 [108]. This disease is caused by mosquito-borne parasitic filarial worms, namely, *Brugia malayi, B. timori*, and *Wuchereria bancrofti*, that are transmitted by some mosquito species. About 90% of lymphatic filariasis cases are caused by *W. bancrofti* and other cases by *Brugia* species [109]. The WHO recommended antihelmintic treatment to eliminate lymphatic filariasis.

Aedes, Culex (predominantly *Cx. quinquefasciatus*) and *Anopheles* species are the principal vectors of lymphatic filariasis causing infections with *W. bancrofti* [110]. *Mansonia* species transmit the disease through *Brugia malayi* and *B. timori*. In 1997, the WHO declared lymphatic filariasis as the second leading disease that causes long-term or permanent disability globally. Integrated vector management programmes have been promoted by the WHO to improve cost-effectiveness, efficacy, ecological soundness, and vector control sustainability [110]. However, the total global burden of this disease continues to grow and its prevalence of endemicity has been confirmed in 76 countries [109].

Lymphatic filariasis (LF) used to be endemic in many parts of Vietnam in the early 1900s, putting millions of people at risk [111,112]. Infections were only caused by *Brugia malayi* and *Wuchereria bancrofti*, in which *B. malayi* made up the majority (over 90%) [111,113]. An estimated 5%–10% of the population became infected in several areas (114). The prevalence of the disease gradually declined to only 1%–3% in endemic areas [114]. In 2018, Vietnam declared the elimination of LF through the support of the WHO and the United States Agency for International Development (USAID) neglected tropical disease (NTD) programme. To maintain these results, Vietnam continues to conduct operational research to monitor and evaluate, having being recognised by the WHO as having eliminated the disease.

4. Other mosquito-borne diseases

Yellow fever

Yellow fever (Flavivirus) is a common mosquito-borne viral disease distributed mostly in South America and Africa countries. The clinical features of this disease range from mild febrile illness to lethal symptoms including haemorrhages and liver damage. Annually, 80%–90% of cases are reported from about 44 Africa countries via epizootic outbreaks [115]. This disease was transmitted to other nations including the United States via slave transport ships in the middle of the 18th century [116]. In 1946, the Pan American Health Organization (PAHO) initiated an eradication campaign to eliminate the vectors causing yellow fever in urban areas of American countries. Unfortunately, this elimination campaign was interrupted and many of the American countries were re-infested by vectors including *Ae. aegypti*, *Ae. africanus*, and Haemagogus janthinomys [116,117]. The main vectors that transmit the yellow fever virus are found to be Aedes sp. in Africa, Haemagogus sp. in South America, and *Ae. aegypti* and *Ae. albopictus* in Brazil. In general, Haemagogus leucocelaenus and Sabethes albiprivus are highly susceptible to the yellow fever virus [117].

Chikungunya disease

Chikungunya is an alphavirus (Togaviridae) that causes chronic musculoskeletal pain and acute fever in humans. In 1953, chikungunya was first reported in Tanzania and was characterised by severe fever and crippling joint pain [118]. Later, this virus became endemic in Africa through frequent sporadic outbreaks that were reported in the Democratic Republic of Congo, Nigeria, Kenya, Uganda, Senegal, South Africa, and Zimbabwe [119]. In a short time, the disease spread to tropical and temperate zones of Asia through mosquito vectors. In 1958, the first outbreak in Asia was recorded in Bangkok and further spread to Cambodia, Malaysia, Vietnam, and Taiwan in the following years [120]. In recent years, chikungunya cases have been reported in Australia, the Caribbean, South America, Europe, the US, the Middle East, and the Pacific region through travellers from affected countries [121,122]. Chikungunya disease is highly debilitating and its epidemics have a significant economic impact. For instance, within nine months of the first chikungunya infection in the Caribbean island of Saint Martin, it had spread to 22 countries by October 2013 [118].

The extensive spread and frequent epidemics causing severe illness have led to the need for effective drug therapy. Generally, analgesics, anti-inflammatory, antiviral, and antipyretics agents are administered for the treatment of chikungunya fever [123]. In 1984, chloroquine phosphate was observed to be an effective therapeutic agent for chronic chikungunya arthritis [124]. Later, various chikungunya vaccines were developed and used, causing fewer side effects [125,126].

The serological characterisation of blood samples collected in epidemic areas in Tanganyika and Thailand reported *Ae. aegypti* to be the major vector of the chikungunya virus [127,128]. Similarly, experimental studies identified *Ae. albopictus* as the potential vector in Indian Ocean islands [128]. The transmission of the chikungunya virus has differed in Africa and Asia. In Africa, the chikungunya virus is sustained in a sylvatic cycle of *Aedes africanus*, *Ae. aegypti* and Ae. furcifer-taylori, while the Asian virus is maintained in a mosquito-human-mosquito cycle of Mansonia and *Culex* sp. [129]. However, the *Anopheles* and *Culex* mosquito vectors need further investigation, as they failed to spread the chikungunya virus experimentally [128].

5. Mosquito population control strategies

The mosquito control strategy in Vietnam has implemented a number of different methods to control vectors. In the 2000s, measures to improve the environment, clear sewers, clear bushes, etc., were an effective strategy in terms of the economy and savings for the state budget. However, once an epidemic occurs, chemical spraying is applied thoroughly and effectively on a large scale and plays a decisive role. In addition, biological methods have also been applied in Vietnam since the 1990s, although with little effect. They are still applied and include methods such as releasing guppies, carp, and tilapia to eat larvae in ponds, lakes and ornamental pots [130]. Currently, the use of Wolbachia-carrying mosquitoes appears to be the most effective means of preventing *Aedes* mosquitoes. Trials in Nha Trang in Khanh Hoa showed a significant reduction in the number of mosquitoes in the population, as well as a reduction in the number of dengue cases [131].

Mechanical methods and environmental improvements

Generally, every stagnation group has appropriate areas to breed, mechanical methods eliminate such place in order to minimise the birth of new spores. In addition, by improving the environment the growth of disease-transmitting insects is interrupted and limited [132]. Environmental improvement aims to be detrimental to the disease-causing species, the ecological imbalance, and maintains this imbalance for as long as possible. For instance, limited ponds, cleaning waste, and opening sewers to limit the growth of flies [133]. Overall, mechanical methods and environmental improvement not only kill the vectors but also prevent them from coming into contact with animals and humans. These methods are promote high awareness, are inexpensive, and very effective, with the biggest advantages of not causing ecological contamination, and leading to sustainable and proactive effects [134].

Chemical methods

Over the last few years, chemical agents such as organochloride, pyrethroids, organophosphorus, thiacloprid, imidacloprid, *N,N*-diethyl-meta-toluamide, thiamethoxam, *p*-menthane-3,8-diol, fendona (α-cypermethrin), and synthetic chrysanthemum drugs have been given top priority for the inhibition of disease-transmitting vectors [134]. These methods not only limit the vector population but also are remarkably effective at controlling outbreaks in Vietnam. The main advantages of these chemical methods are that they are quick-acting and are highly effective, even on large areas. However, this method is limited by the chemical resistance of vectors and environmental pollution [135].

Biological methods

In this method, either natural enemies of insects are used to destroy vectors (prey) or disease-causing organisms are used to infect with mosquitoes diseases [136]. For instance, the use of fish to kill larvae, and the release of *Toxorhynchites* mosquito larvae and/or microbes (such as *Bacillus thuringiensis*, *MESOCYCLOPS*) to eat the larvae of *Aedes, Anopheles, Ceolomomyces, Culex, Culicinomyces, Entomophthora, Lagenidium* and *Tolypocladium* [130,131,137–139]. Biological methods have the advantage of not polluting the environment and are non-toxic to animals and humans. On the other hand, this method is less effective [134].

Genetic methods

Currently, genetically modified mosquitoes are using as a tool to kill the mosquitoes that transmit dengue fever in Malaysia, Brazil, and Vietnam [140]. In this method, male mosquitoes are genetically engineered and released into the wild to mate with female mosquitoes. The larvae produced after mating with these modified mosquitoes will eventually moult or die at the four instar [141]. This method has been shown to be effective in controlling the transmission of dengue fever. However, genetic methods remain controversial with regards to the emergence of novel species [140].

6. An emerging method for the rapid and reliable identification of mosquito species

Mosquito-borne diseases are a major public health concern worldwide. For effective mosquito control, their accurate identification is a crucial step in differentiating mosquito vectors from non-vectors. Morphological identification is the "gold standard" method, which is widely used for discriminating between mosquitoes using their external characteristics. However, there are several drawbacks to this traditional method, such as the need for standard taxonomic keys, expert entomology knowledge, and the ability to classify species belonging to sibling and cryptic groups [142,143]. Interestingly, most of the significant malaria vectors in the SEA region are members of complexes in which the species are similar or indistinguishable using external morphology. Molecular biology methods such as polymerase chain reaction (PCR), enzyme electrophoresis, and DNA sequencing, have been used to distinguish between homogeneous species [144,145]. Nevertheless, these techniques are also limited due to being time-consuming, exorbitantly priced, and requiring primer-specific targeting for certain species [146–148].

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has revolutionised the field of clinical microbiology and mycology [146]. Recently, MALDI-TOF MS has been proposed as an alternative and innovative method of overcoming the drawbacks of the above two methods in medical entomology to identify arthropods [148]. This approach has routinely been applied in our laboratory for the rapid and accurate identification of arthropods, including mosquitoes at almost all stages such as eggs, larvae, and adults [149–151]. MALDI-TOF MS is an easy-to-use, time-saving, and affordable method compared to earlier methods, and a device may be purchased for medical microbiology and then used in entomology. Its operation is based on the identification of acidic extraction of proteins from an organ of interest. The mass/charge ratio of each protein is measured as it passes across an electric field after propulsion of the protein molecules from ultraviolet laser desorption. The mass/charge ratio of the proteins generated is unique protein mass spectrum of the specific sample known as the "protein signature" for reliable species identification. This spectrum is then compared to a database of reference profiles containing the spectra of species that have been officially validated using morphology and molecular techniques.

In Vietnam, MALDI-TOF MS was firstly applied to identify mosquitoes collected in eight areas of the Vietnam Central Highlands, including 22 mosquito species identified from extracted proteins from the legs. In particular, MALDI-TOF MS is able to distinguish between closely related mosquito species, which are impossible to classify using morphology, as was the case of the *Anopheles minimu*s [152,153] and *Culex pipiens* complexes [149], which have been reported as being challenging to distinguish from others using morphology and molecular techniques [149,154–156]. This innovative technique is capable of successfully identifying Vietnamese mosquitoes at the species level, as well as within the sibling and complex groups. It indicates that MALDI-TOF MS could be used to prevent mosquito misidentification by morphology.

7. Conclusion and perspectives

Recently, the collection of mosquitoes in Vietnam has mainly focussed on vector species related to the transmission of infectious diseases that cause global health problems. Eighty-nine mosquito species were recognised prior to 1970 and 192 species have been described from 1970 to the present. [36]. However, the checklist of Vietnamese mosquitoes is far from being complete as long as further investigations on mosquitoes are carried out. Molecular biology tools have proven to greatly enhance the accurate identification of the microorganisms known to be associated with mosquitoes such as bacteria, protozoa, and arbovirus [158–160]. These methods not only confirm the morphological identification but also identify species and contribute to the detection of mosquito-borne microorganisms.

A large number of vectors of the genera *Aedes, Anopheles,* and *Culex* have been identified as the mosquitoes that transmit diseases caused by parasites, bacteria, and viruses. Interestingly, outbreaks of dengue, Zika, and Japanese encephalitis have occurred in Vietnam and around the world where are *Aedes* spp. mosquitoes are prevalent. Following these viruses, we can hypothesise that a further pathogen transmitted by *Anophele*s and *Aedes* spp. mosquitoes probably appears in Vietnam as *Rickettsia felis* [161]. In fact, an increasing number of reports have implicated *R. felis*, an obligate intracellular bacterial pathogen that is able to cause disease in humans [161–163]. The first report of *R. felis* as a human pathogen detected it in two acute undifferentiated fever (AUF) patients and one afebrile adult patient in central Vietnam [164]. The cat flea *Ctenocephalides felis* was first considered to be the only confirmed vector of *R. felis* [161]. Nevertheless, we recently discovered that *Anopheles gambiae,* a major malaria vector in sub-Saharan Africa, was a potential vector for *R. felis* [163]. Additionally, *R. felis* has been detected in *Anopheles sinensis*, *Aedes albopictus*, and *Culex pipiens pallens* mosquitoes from China, where these vectors bite human beings [165]. Therefore, further studies are needed to determine the range of mosquitoes hosting *R. felis* in Vietnam.

468

References

1. Becker, N.; Petrić, D.; Zgomba, M.; Boase, C.; Madon, M.; Dahl, C.; *et al*. Mosquitoes and Their Control: Second Edition. *Springer-Verlag Berlin Heidelberg*. 2010. 1–577 p.

2. Gubler, D.J. Dengue, Urbanization and globalization: The unholy trinity of the 21 st century. *Tropical Medicine and Health*. 2011;39(4 Suppl):3–11.

3. Tandina, F.; Niaré, S.; Laroche, M.; Koné, A.K.; Diarra, A.Z.; Ongoiba, A.; *et al*. Using MALDI-TOF MS to identify mosquitoes collected in Mali and their blood meals. *Parasitology*. 2018;145(9):1170–82.

4. Azari-Hamidian, S.; Norouzi, B.; Harbach, R.E. A detailed review of the mosquitoes (Diptera: Culicidae) of Iran and their medical and veterinary importance. *Acta Trop*. 2019 Jun;194:106–22.

5. Tolle, M.A. Mosquito-borne diseases. *Curr Probl Pediatr Adolesc Health Care*. 2009 Apr;39(4):97–140.

6. World Health Organization. Vector-borne diseases. 2020. Available online: https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases (accessed on 28 Jun 2021).

7. Nguyen-Tien, T.; Lundkvist, Å.; Lindahl, J. Urban transmission of mosquito-borne flaviviruses–a review of the risk for humans in Vietnam. *Infection Ecology and Epidemiology*. Taylor and Francis Ltd.; 2019,9.

8. Manh, N.D.; Thanh, N.V.; Quang, H.H.; Van, N.T.T.; San, N.N.; Phong, N.C.; *et al*. Pyronaridine-Artesunate (Pyramax) for Treatment of Artemisinin- and Piperaquine-Resistant *Plasmodium falciparum* in the Central Highlands of Vietnam. *Antimicrob Agents Chemother*. 2021 Nov 17;65(12):e0027621.

9. Pham, K.;Hotez. P.J. Vietnam: Neglected tropical diseases in an emerging and accelerating economy. *PLoS Negl Trop Dis*. 2022 Feb 17;16(2):e0010140.

10. Do, M.C.; Beebe, N.W.; Thi, V.V.N.; Le, Q.T.; Lein, C.T.; Van, N.D.; *et al*. Vectors and malaria transmission in deforested, rural communities in north-central Vietnam. *Malar J*. 2010 Sep 16;9:259.

11. Ohba, S.Y.; Huynh, T.T.T.; Le, L.L.; Ngoc, H.T.; Hoang, S.L.; Takagi, M. Mosquitoes and their potential predators in rice agroecosystems of the Mekong Delta, southern Vietnam. *J Am Mosq Control Assoc*. 2011 Dec;27(4):384–92.

12. Quang, B.P.; Hong, H.Q.; Thanh, T.D.; Thanh, L.D.; Quang, N.T.; Van, T.H.; *et al*. Pyronaridine-artesunate Efficacy and Safety in Uncomplicated *Plasmodium falciparum* Malaria in Areas of Artemisinin-resistant Falciparum in Viet Nam (2017-2018). *Clin Infect Dis*. 2020 May 6;70(10):2187–95.

13. Nguyen, T.Q.; Nguyen, M.D.; Pham, V.X.; Ro, H.M.; Edstein, M.D.; Chow, W.K.; *et al*. Entomological survey in two communes with residual malaria transmission in Gia Lai Province in the central highlands of Vietnam. *Malar J*. 2021 Dec;20(1):403.

14. Canavati, S.E.; Kelly, G.C.; Vo, T.H.; Tran, L.K.; Ngo, T.D.; Tran, D.T.; *et al*. Mosquito Net Ownership, Utilization, and Preferences among Mobile and Migrant Populations Sleeping in Forests and Farms in Central Vietnam: A Cross-Sectional Study. *Am J Trop Med Hyg*. 2021 May;104(5):1917–24.

15. Quang, H.H.; Chavchich, M.; Trinh, N.T.M.; Edgel, K.A.; Edstein, M.D.; Martin, N.J. Multidrug-Resistant *Plasmodium falciparum* Parasites in the Central Highlands of Vietnam Jeopardize Malaria Control and Elimination Strategies. *Antimicrob Agents Chemother*. 2021 Mar 18;65(4):e01639-20.

16. Peeters, G.K.; Xuan, X.N.; Van, B.W.; Duc, T.N.; Ribera, J.M.; Ba, N.T.; *et al*. Low perception of malaria risk among the Ra-glai ethnic minority in south-central Vietnam: implications for forest malaria control. *Malar J*. 2010 Jan 20;9:23.

17. Thanh, P.V.; Van, H.N.; Van, V.N.; Van, M.C.; Obsomer, V.; Rosanas-Urgell A.; et al. Epidemiology of forest malaria in Central Vietnam: the hidden parasite reservoir. *Malar J*. 2015.

18. Hii, J.; Rueda, L.M. Malaria vectors in the Greater Mekong Subregion: overview of malaria vectors and remaining challenges. *Southeast Asian J Trop Med Public Health*. 2013;44 Suppl 1:73–165; discussion 306-307.

509   19.   Verhaeghen, K.; Van, B.W.; Trung, H.D.; Sochantha, T.; Coosemans, M. Absence of knockdown resistance suggests metabolic resistance
510   in the main malaria vectors of the Mekong region. *Malar J*. 2009 Apr 28;8:84.

511   20.   World Health Organization (WHO). World malaria report 2011. Geneva: WHO, 2011a. Available online:
512   http://www.who.int/malaria/world_malaria_report_2011/9789241564403_eng.pdf (accessed on 15 Sep 2021).

513   21.   World Health Organization. National malaria programme review - Viet Nam. 2018. Available online:
514   https://www.who.int/publications/i/item/9789290618423 (accessed on 15 Sept 2021).

515   22.   Müller, O.; Lu, G.Y.; Seidlein, L. Geographic expansion of artemisinin resistance. *J Travel Med*. 2019 Jun 1;26(4):taz030.

516   23.   Ataíde, R.; Powell, R.; Moore, K.; McLean, A.; Phyo, A.P.; Nair, S.; *et al*. Declining Transmission and Immunity to Malaria and Emerging
517   Artemisinin Resistance in Thailand: A Longitudinal Study. *J Infect Dis*. 2017 Sep 15;216(6):723–31.

518   24.   Moncayo, A.C.; Fernandez, Z.; Ortiz, D.; Diallo, M.; Sall, A.; Hartman, S.; *et al*. Dengue Emergence and Adaptation to Peridomestic
519   Mosquitoes. *Emerg Infect Dis*. 2004 Oct;10(10):1790–6.

520   25.   Nguyen, L.T.; Le, H.X.; Nguyen, D.T.; Ho, H.Q.; Chuang, T.W. Impact of Climate Variability and Abundance of Mosquitoes on Dengue
521   Transmission in Central Vietnam. *Int J Environ Res Public Health*. 2020 Apr;17(7):2453.

522   26.   World Health Organization. Comprehensive Guidelines for Prevention and Control of Dengue and Dengue Haemorrhagic Fever; WHO
523   Regional Publication for South-Asia: New Delhi, India. 2011;159–168. Available online: https://apps.who.int/iris/handle/10665/204894
524   (accessed on 28 Sep 2021).

525   27.   World Bank Group, Asian Development Bank. Climate Risk Country Profile: Vietnam. World Bank; 2021 . Available online:
526   http://elibrary.worldbank.org/doi/book/10.1596/36367 (accessed on 1 Arp 2022).

527   28.   Chapot, L.; Nguyen-Tien, T.; Pham-Thanh, L.; Nguyen-Viet, H.; Craven, L.; Lindahl, J.F. A mixed-methods approach to understanding
528   knowledge of mosquito-borne infections and barriers for protection in Hanoi, Vietnam. *Tropical Medicine and Infectious Disease*. 2020
529   May 1;5(2):66.

530   29.   Parrish, D.W. The Occurrence and Known Human-Disease Relationships of Mosquitoes on usaf Installations in The Republic of Vietnam.
531   *Epidemiological Flight* (5TH) APO San Francisco 96528. Available online: https://apps.dtic.mil/sti/citations/AD0677195 (accessed on 27
532   Dec 2021).

533   30.   Reinert, J.F. Contributions to the Mosquito Fauna of Southeast Asia. V. Genus *Aedes*, Subgenus Diceromyia Theobald in Southeast Asia.
534   Walter Reed Army Inst of Research Washington DC; 1970. Available online: https://apps.dtic.mil/sti/citations/ADA510732 (accessed on
535   27 Dec 2021).

536   31.   Tyson, W. Contributions to the Mosquito Fauna of Southeast Asia. VIII. Genus *Aedes*, Subgenus Mucidus Theobald in Southeast Asia.
537   *Contributions of the American Entomological Institute* (Ann Arbor). 1970 Jan 1;6:53.

538   32.   Thoa, N.; Vien, N.; Mai, T.; Xuan, N. Japanese encephalitis vectors: isolation of virus from culicine mosquitoes in the Saigon area.
539   *Southeast Asian Journal of Tropical Medicine and Public Health*. 1974;5(3):408–12.

540   33.   Thuan, L.K. Anopheles meigen mosquito (diptera : culicidae) transmits malaria in central Vietnam (latitude 11-16 North). [Hanoi]:
541   Ministry of Health; 1988.

542   34.   Vu, D.H. Muoi Aedes Meigen (Cilidae, Diptera) o mien Bac Viet Nam. Luan an pho tien si Khoa hoc Sinh hoc. Truong Dai hoc Tong hop
543   Ha Noi. 1984. Available online: http://luanan.nlv.gov.vn/luanan?a=d&d=TTjFvmmrdCnK1984.1.1# (accessed on 2 Aug 2022).

544   35.   Bui, P.; Darsie, R.F. Tentative Checklist of the Mosquitoes of Vietnam Employing New Classification for Tribe Aedini (Diptera,
545   Culicidae). *Journal of the American Mosquito Control Association*. 2008 Jun;24(2):187–93.

546   36.   Nguyen, V.D. Checklist of the known species of culicidae found in vietnam. Hoi Nghi Khoa Hoc Toan Quoc Ve Sinh Thai va Tai Nguyen
547   Sinh Vat Lan thu 6. 2016. Available online: http://iebr.ac.vn/database/HNTQ6/504.pdf (accessed on 5 May 2021).

548   37.   Reinert, J.F. *Aedes* Consonensis, a New Species of the Subgenus Neomacleaya from South Vietnam (Diptera: Culicidae). Walter reed
549   army inst of research Washington DC; 1973. Available online: https://apps.dtic.mil/sti/citations/ADA511213 (accessed on 23 Aug 2022).

550   38.   Harbach, R.E. The Culicidae (Diptera): a review of taxonomy, classification and phylogeny*. *Zootaxa*. 2007 Dec 21;1668(1):591–638.

551 39. Chitturi, B.R.; Chanti, D.B.; Tatipamula, V.B.; Dudem, S.; Kalivend, S.V.; Tuniki, V.R.; *et al*. Isolation, structural assignment and
552   synthesis of (S,E)-2-methyloctyl 3-(4-methoxyphenyl) propenoate from the marine soft coral Sarcophyton ehrenbergi. *Natural Product*
553   *Research*. 2015 Jan 2;29(1):70–6.

554 40. Snow, R.W.; Guerra, C.A.; Noor, A.M.; Myint, H.Y.; Hay, S.I. The global distribution of clinical episodes of *Plasmodium falciparum*
555   malaria. *Nature*. 2005 Mar 10;434(7030):214–7.

556 41. Hung, L.Q.; de Vries, P.J.; Giao, P.T.; Nam, N.V.; Binh, T.Q.; Chong, M.T. Control of malaria: a successful experience from Viet Nam.
557   *Bulletin of the World Health Organization*. 2002;7.

558 42. Martin, N.J.; Nam, V.S.; Lover, A.A.; Phong, T.V.; Tu, T.C.; Mendenhall, I.H. The impact of transfluthrin on the spatial repellency of the
559   primary malaria mosquito vectors in Vietnam: *Anopheles dirus* and *Anopheles minimus*. *Malar J*. 2020 Jan 6;19:9.

560 43. Pham, V.T. Epidemiology of *Plasmodium vivax* malaria in central vietnam. 2018. Available online:
561   https://dial.uclouvain.be/pr/boreal/en/object/boreal%3A202970/ datastream/PDF_ 01/view (17 Sep 2021).

562 44. Lynch, C.; Hewitt, S. Malaria in the Asia-Pacific: Burden, success and challenges. In 2012.

563 45. Vu, V.H.; Nguyen, T.H.B.; Vu, D.C. Nghien cuu thanh phan loai, phan bo, tap tinh, vai tro truyen sot ret cua muoi *Anopheles* va hieu luc
564   cua kem xua, huong diet muoi NIMPE tai huyen dong xuan, tinh Phu Yen, 2017 - 2019. Available online:
565   http://nimpe.vn/userfiles/file/DaotaoSDH/Vu%20Viet%20Hung/Luan_an_Vu_Viet_Hung.pdf (accessed on 8 Oct 2021).

566 46. Ngo, K.K.; Nguyen, X.Q.; Nguyen, V.C.; Nguyen, T.M.D. Vector abundance and species composition of *Anopheles* mosquito in Central
567   Region and Central West Highlands, Viet Nam. *International Journal of Health and Clinical Research*. 2018;8(1(1)):22–9.

568 47. Edwards, H.M. Chinh, V.D.; Le, D.B.; Thanh, P.V.; Thang, N.D.; Trang, D.M.; *et al*. Characterising residual malaria transmission in
569   forested areas with low coverage of core vector control in central Viet Nam. *Parasit Vectors*. 2019 Sep 18;12(1):454.

570 48. Van, B.W.; Trung, H.D.; Thuan, L.K.; Sochantha, T.; Socheat, D.; Sumrandee, C.; *et al*. The insecticide resistance status of malaria vectors
571   in the Mekong region. *Malar J*. 2008 Jun 5;7:102.

572 49. Abeyasinghe, R.; Vu, D.C.; Tran, C.D.; Habib, N.; Hewitt, S.; Nguyen, M.H.; *et al*. National malaria programme review - Viet Nam. 2018.
573   Available noline: https://www.who.int/publications/i/item/9789290618423(accessed on 17 Mar 2022).

574 50. Obsomer, V.; Defourny, P.; Coosemans, M. The *Anopheles dirus* complex: spatial distribution and environmental drivers. *Malar J*. 2007
575   Mar 6;6:26.

576 51. Takano, K.T.; Nguyen, N.T.H.; Nguyen, B.T.H.; Sunahara, T.; Yasunami, M.; Nguyen, M.D.; *et al*. Partial mitochondrial DNA sequences
577   suggest the existence of a cryptic species within the Leucosphyrus group of the genus *Anopheles* (Diptera: Culicidae), forest malaria
578   vectors, in northern Vietnam. *Parasit Vectors*. 2010 Apr 30;3:41.

579 52. Van, L.B.; Allen, G.; Brauny, M.; Linh, L.T.K.; Pallerla, S.R.; Huyen, T.T.T.; *et al*. Molecular surveillance and temporal monitoring of
580   malaria parasites in focal Vietnamese provinces. *Malar J*. 2020 Dec 31;19(1):458.

581 53. Kawamoto, F.; Miyake, H.; Kaneko, O.; Kimura, M.; Nguyen, T.D.; Nguyen, T.D.; *et al*. Sequence variation in the 18S rRNA gene, a
582   target for PCR-based malaria diagnosis, in *Plasmodium ovale* from southern Vietnam. *J Clin Microbiol*. 1996 Sep;34(9):2287–9.

583 54. Eede, P.; Van, H.; Overmeir, C.; Vythilingam, I.; Duc, N.; Hung, L.; *et al*. Human *Plasmodium knowlesi* infections in young children in
584   central Vietnam. *Malaria journal*. 2009 Oct 30;8:249.

585 55. Maeno, Y. Molecular epidemiology of mosquitoes for the transmission of forest malaria in south-central Vietnam. Trop Med Health.
586   2017;45. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5637239/ (accessed on 9 Oct 2021).

587 56. Marchand, R.P.; Culleton, R.; Maeno, Y.; Quang, N.T.; Nakazawa, S. Co-infections of Plasmodium knowlesi, P. falciparum, and *P. vivax*
588   among Humans and *Anopheles dirus* Mosquitoes, Southern Vietnam. *Emerg Infect Dis*. 2011 Jul;17(7):1232–9.

589 57. Harrison B.A. Medical entomology studies. XIII. The myzomyia series of Anopheles (Cellia) in Thailand, with emphasis on intra-
590   interspecific variations (Diptera: Culicidae). *Contributions of the American Entomological Institute*. 1979; Available online:
591   https://scholar.google.com/scholar_lookup?title= (accessed on 22 Apr 2022).

592 58. Dusfour, I.; Blondeau, J.; Harbach, R.E.; Vythilingham, I.; Baimai, V.; Trung, H.D.; *et al*. Polymerase Chain Reaction Identification of
593 Three Members of the *Anopheles sundaicus* (Diptera: Culicidae) Complex, *Malaria Vectors in Southeast Asia*. 2007 Sep 1;44(5):723–
594 31.

595 59. World Health Organization. Dengue and severe dengue. 2021. Available online: https://www.who.int/news-room/fact-
596 sheets/detail/dengue-and-severe-dengue (accessed on 3 Jul 2021).

597 60. Mustafa, M.S.; Rasotgi, V.; Jain, S.; Gupta, V. Discovery of fifth serotype of dengue virus (denv-5): A new public health dilemma in
598 dengue control.Medical Journal Armed Forces India. *Medical Journal Armed Forces India*. 2015, 71, p. 67–70.

599 61. Vietnam reports big increase in dengue fever in 2019 - Outbreak News Today. Available online: http://outbreaknewstoday.com/vietnam-
600 reports-big-increase-in-dengue-fever-in-2019/ (accessed on 10 Jul 2021).

601 62. Quyen, D.L.; Le, N.T; Van-Anh, C.T.; Nguyen, N.B.; Van, H.D.; Montgomery, J.L.; *et al*. Epidemiological, serological, and virological
602 features of dengue in Nha Trang City, Vietnam. *American Journal of Tropical Medicine and Hygiene*. 2018 Jan 8;98(2):402–9.

603 63. Lee, H.S, Nguyen-Viet, H.; Nam, V.S.; Lee, M.; Won, S.; Duc, P.P; *et al*. Seasonal patterns of dengue fever and associated climate factors
604 in 4 provinces in Vietnam from 1994 to 2013. *BMC Infectious Diseases*. 2017 Mar 20;17(1):1–9.

605 64. Rabaa, M.A.; Simmons, C.P.; Fox, A.; Le, M.Q.; Nguyen, T.T.T.; Le, H.Y; *et al*. Dengue Virus in Sub-tropical Northern and Central Viet
606 Nam: Population Immunity and Climate Shape Patterns of Viral Invasion and Maintenance. *PLoS Neglected Tropical Diseases*.
607 2013;7(12):2581.

608 65. NewsDesk. Vietnam records 15K additional dengue cases in 10 days, Country total now 92,000. Outbreak News Today. 2022. Available
609 online: http://outbreaknewstoday.com/vietnam-records-15k-additional-dengue-cases-in-10-days-country-total-now-92000/ (accessed on 7
610 Jul 2022).

611 66. SGGP. Dengue toll rises to 36 in Vietnam, case count now over 92,000. *SGGP* English Edition. Available online: https://sggpnews.org.vn/
612 content/OTU5Mzk=.html (accessed on 7 Jul 2022).

613 67. VietnamPlus. Thanh pho Ho Chi Minh: 9 truong hop tu vong do sot xuat huyet | Y te | Vietnam+ (VietnamPlus) [Internet]. VietnamPlus.
614 2022 [cited 2022 Jul 7]. Available from: https://www.vietnamplus.vn/thanh-pho-ho-chi-minh-9-truong-hop-tu-vong-do-sot-xuat-
615 huyet/799612.vnp

616 68. Vietnam Posts English. Binh Duong becomes a hot spot for dengue fever. Vietnam Posts English. 2022. Available online:
617 https://vietnam.postsen.com/news/38983/Binh-Duong-becomes-a-hot-spot-for-dengue-fever.html (accessed on 7 Jul 2022).

618 69. VNS. Dengue fever rises in Dong Nai - Viet Nam | ReliefWeb. 2022. Available online: https://reliefweb.int/report/viet-nam/dengue-fever-
619 rises-dong-nai (accessed on 7 Jul 2022).

620 70. Lambrechts, L.; Scott, T.W.; Gubler, D.J. Consequences of the expanding global distribution of *Aedes albopictus* for dengue virus
621 transmission. *PLoS Neglected Tropical Diseases. Public Library of Science*; 2010. p. e646. Vol. 4

622 71. Chen, L.H.; Hamer, D.H. Zika Virus: Rapid spread in the western hemisphere. *Annals of Internal Medicine*. 2016 May 3;164(9):613–5.

623 72. Dick, G.W.; Kitchen, S.F.; Haddow, A.J. Zika virus. I. Isolations and serological specificity. *Transactions of the Royal Society of Tropical*
624 *Medicine and Hygiene*. 1952;46(5):509–20.

625 73. Musso, D.; Gubler, D.J. Zika Virus. *Clin Microbiol Rev*. 2016 Jul;29(3):487–524.

626 74. MacNamara, F.N. Zika virus : A report on three cases of human infection during an epidemic of jaundice in Nigeria. *Transactions of The*
627 *Royal Society of Tropical Medicine and Hygiene*. 1954 Mar 1;48(2):139–45.

628 75. Hayes, E.B. Zika Virus Outside Africa. *Emerging Infectious Diseases*. 2009 Sep;15(9):1347.

629 76. Musso, D.; Ko, A.I.; Baud, D. Zika Virus Infection — After the Pandemic. Longo DL, editor. *N Engl J Med*. 2019 Oct 10;381(15):1444–
630 57.

631 77. Weaver, S.C.; Costa, F.; Garcia-Blanco, M.A.; Ko, A.I.; Ribeiro, G.S.; Saade, G.; *et al*. Zika virus: History, emergence, biology, and
632 prospects for control. *Antiviral Research*. 2016 Jun 1;130:69–80.

633 78. Hamel, R.; Liégeois, F; Wichit, S.; Pompon, J.; Diop, F.; Talignani, L; *et al.* Zika virus: epidemiology, clinical features and host-virus
634   interactions. *Microbes and Infection*. 2016 Jul 1;18(7–8):441–9.

635 79. Musso, D.; Bossin, H.; Mallet, H.P.; Besnard, M.; Broult, J; Baudouin, L.; *et al*. Zika virus in French Polynesia 2013–14: anatomy of a
636   completed outbreak. *The Lancet Infectious Diseases*. 2018 May;18(5):e172–82.

637 80. Vasilakis, N.; Weaver, S.C. Flavivirus transmission focusing on Zika. *Current Opinion in Virology*. 2017 Feb 1;22:30–5.

638 81. Boyer, S.; Calvez, E.; Chouin-Carneiro, T.; Diallo, D.; Failloux, A.B. An overview of mosquito vectors of Zika virus. *Microbes and*
639   *Infection*. 2018 Dec 1;20(11–12):646–60.

640 82. Jamali, M.S.R.; Bayrami, S.; Jamali, M.S.; Golrokhi, R.; Golsoorat, P.F.; Seyed, A.S.A. Zika virus: A review of literature. *Asian Pacific*
641   *Journal of Tropical Biomedicine*. 2016 Dec 1;6(12):989–94.

642 83. Chan, J.F.W.; Choi, G.K.Y.; Yip, C.C.Y.; Cheng, V.C.C.; Yuen K.Y. Zika fever and congenital Zika syndrome: An unexpected emerging
643   arboviral disease. *Journal of Infection*. 2016 May 1;72(5):507–24.

644 84. Wilder-Smith, A.; Chang, C.R.; Leong, W.Y. Zika in travellers 1947–2017: a systematic review. *Journal of Travel Medicine*. 2018 Jan
645   1;25(1):1947–2017.

646 85. Quyen, N.T.H.; Kien, T.H.; Rabaa, M.; Tuan, N.M.; Vi, T.T.; Tan, L.V.; *et al*. Chikungunya and Zika Virus Cases Detected against a
647   Backdrop of Endemic Dengue Transmission in Vietnam. *The American Journal of Tropical Medicine and Hygiene*. 2017 Jul 12;97(1):146–
648   50.

649 86. Bui, T.C.; Huynh, X.V.; Nguyen, H.; Nguyen, K.T.; Nguyen, C.D.; Pham, N.D. Zika virus outbreaks and treatment in pregnant women in
650   Ho Chi Minh City, Vietnam. *International Journal of Gynecology & Obstetrics*. 2018 Jun 1;141(3):390–1.

651 87. Chu, D.T.; Ngoc, V.T.N.; Tao, Y. Zika virus infection in Vietnam: current epidemic, strain origin, spreading risk, and perspective. *Eur J*
652   *Clin Microbiol Infect Dis*. 2017 Nov;36(11):2041–2.

653 88. Nguyen-Tien, T. Probandari, A.; Ahmad, R.A. Barriers to Engaging Communities in a Dengue Vector Control Program: An
654   Implementation Research in an Urban Area in Hanoi City, Vietnam. *The American Journal of Tropical Medicine and Hygiene*. 2019 Jan
655   14;100(4):964–73.

656 89. Wang, H.; Liang, G. Epidemiology of Japanese encephalitis: past, present, and future prospects. *Therapeutics and Clinical Risk*
657   *Management*. 2015 Mar 19;11:435.

658 90. Campbell, G.L.; Hills, S.L.; Fischer, M.; Jacobson, J.A.; Hoke, C.H.; Hombach, J.M.; et al. Estimated global incidence of Japanese
659   encephalitis: a systematic review. *Bulletin of the World Health Organization*. 2011;89(10):766.

660 91. Burns, K.F. Congenital Japanese B Encephalitis Infection of Swine: https://doi.org/103181/00379727-75-18285. 2016 Nov 8;75(2):621–
661   5.

662 92. Erlanger, T.E.; Weiss, S.; Keiser, J.; Utzinger, J.; Wiedenmayer, K. Past, Present, and Future of Japanese Encephalitis. 2009 - Emerging
663   Infectious Diseases journal - CDC. *Emerging Infectious Diseases*. 2009 Jan;15(1):1–7.

664 93. Endy, T.P.; Nisalak, A. Japanese Encephalitis Virus: Ecology and Epidemiology. *Current Topics in Microbiology and Immunology*.
665   2002;267:11–48.

666 94. Liu, W.; Gibbons, R.V;, Karik, K.; Clemens, J.D.; Nisalak, A.; Marks, F.; *et al*. Risk factors for Japanese encephalitis: a case-control study.
667   *Epidemiology & Infection*. 2010 Sep;138(9):1292–7.

668 95. Keiser, J; Maltese, M.F.; Erlanger, T.E.; Bos, R..; Tanner, M.; Singer, B.H.; *et al*. Effect of irrigated rice agriculture on Japanese
669   encephalitis, including challenges and opportunities for integrated vector management. *Acta Tropica*. 2005 Jul 1;95(1):40–57.

670 96. Chen, W.J.; Dong, C.F.; Chiou, L.Y.; Chuang, W.L. Potential Role of *Armigeres subalbatus* (Diptera: Culicidae) in the Transmission of
671   Japanese Encephalitis Virus in the Absence of Rice Culture on Liu-Chiu Islet, Taiwan. *Journal of Medical Entomology*. 2000 Jan
672   1;37(1):108–13.

97. Thenmozhi, V.; Rajendran, R.; Ayanar, K.; Manavalan, R.; Tyagi, B.K. Long-term study of Japanese encephalitis virus infection in *Anopheles subpictus* in Cuddalore district, Tamil Nadu, South India. *Tropical Medicine & International Health*. 2006 Mar 1;11(3):288–93.

98. Epstein, P.R. West Nile virus and the climate. *Journal of Urban Health*. 2001 78:2. 2001;78(2):367–71.

99. Whelan, P; Hayes, G.; Tucker, G.; Carter, J.; Wilson, A.; Haigh, B. The detection of exotic mosquitoes in the Northern Territory of Australia. *Arbovirus Research in Australia*. 2001;8:395–404.

100. Yen, N.T.; Duffy, M.R.; Hong, N.M.; Hien, N.T.; Fischer, M.; Hills, S.L. Surveillance for Japanese Encephalitis in Vietnam, 1998–2007. *The American Journal of Tropical Medicine and Hygiene*. 2010 Oct 5;83(4):816–9.

101. Kuwata, R.; Nga, P.T.; Yen, N.T.; Hoshino, K.; Isawa, H.; Higa, Y.; *et al*. Surveillance of Japanese Encephalitis Virus Infection in Mosquitoes in Vietnam from 2006 to 2008. *The American Journal of Tropical Medicine and Hygiene*. 2013 Apr 3;88(4):681–8.

102. Hasegawa, M.; Tuno, N.; Yen, N.;, Nam, V.S.; Takagi, M. Influence of the Distribution of Host Species on Adult Abundance of Japanese Encephalitis Vectors—*Culex vishnui* Subgroup and *Culex gelidus*—in a Rice-Cultivating Village in Northern Vietnam. *The American Journal of Tropical Medicine and Hygiene*. 2008 Jan 1;78(1):159–68.

103. Lindahl, J.; Chirico, J.; Boqvist, S.; Thu, H.T.V.; Magnusson, U. Occurrence of Japanese Encephalitis Virus Mosquito Vectors in Relation to Urban Pig Holdings. *The American Journal of Tropical Medicine and Hygiene*. 2012 Dec 5;87(6):1076–82.

104. Ha, T.V.; Kim, W.; Nguyen-Tien, T.; Lindahl, J.; Nguyen-Viet, H.; Thi, N.Q.; *et al*. Spatial distribution of Culex mosquito abundance and associated risk factors in Hanoi, Vietnam. *PLoS Negl Trop Dis*. 2021 Jun 21;15(6):e0009497.

105. Ohba, S.Y.; Van, S.N.; Van-Anh, D.T.; Nguyen, Y.T.; Takagi, M. Study of mosquito fauna in rice ecosystems around Hanoi, northern Vietnam. *Acta Trop*. 2015 Feb;142:89–95.

106. Deshpande, A.; Miller-Petrie, M.K.; Johnson, K.B.; Abdoli, A.; Abrigo, M.R.M.; Adekanmbi, V.; *et al*. The global distribution of lymphatic filariasis, 2000–18: a geospatial analysis. *The Lancet Global Health*. 2020 Sep 1;8(9):e1186–94.

107. Ottesen, E.A.; Duke, B.O.; Karam, M.; Behbehani, K. Strategies and tools for the control/elimination of lymphatic filariasis. *Bulletin of the World Health Organization*. 1997;75(6):491.

108. Debrah, L.B.; Mohammed, A.; Osei-Mensah, J.; Mubarik, Y.; Agbenyega, O.; Ayisi-Boateng, N.K.; *et al*. Morbidity management and surveillance of lymphatic filariasis disease and acute dermatolymphangioadenitis attacks using a mobile phone-based tool by community health volunteers in Ghana. *PLOS Neglected Tropical Diseases*. 2020 Nov 1;14(11):e0008839.

109. Melrose, W.D. Lymphatic filariasis: new insights into an old disease. *International Journal for Parasitology*. 2002 Jul 1;32(8):947–60.

110. Van-den, B.H.; Kelly-Hope, L.A.; Lindsay, S.W. Malaria and lymphatic filariasis: the case for integrated vector management. *The Lancet Infectious Diseases*. 2013 Jan 1;13(1):89–94.

111. Meyrowitsch, D.W.; Toan, N.;, Hao, H.T.; Dan, N.T.; Michael, E. A review of the present status of lymphatic filariasis in Vietnam. *Acta Tropica*. 1998 Jul;70(3):335–47.

112. WHO. Three more countries eliminate lymphatic filariasis. Available online: https://www.who.int/westernpacific/news/item/08-10-2018-three-more-countries-eliminate-lymphatic-filariasis (accessed on 24 May 2022).

113. Tran VD. Benh giun chi bach huyet – Benh Ky Sinh Trung. Available online: https://benhkysinhtrung.vn/benh-giun-chi-bach-huyet/ (accessed on 24 May 2022).

114. General Departerment of Preventive Medicine. Le cong bo loai tru benh giun chi Bach huyet tai Vietnam 2019. Available online: https://vncdc.gov.vn/le-cong-bo-loai-tru-benh-giun-chi-bach-huyet-tai-viet-nam-nd14898.html (accessed on 24 May 2022).

115. Monath, T.P.; Vasconcelos, P.F.C. Yellow fever. *Journal of Clinical Virology*. 2015 Mar 1;64:160–73.

116. Bryan, C.S.; Moss, S.W.; Kahn, R.J. Yellow fever in the Americas. *Infectious Disease Clinics of North America*. 2004 Jun 1;18(2):275–92.

117. Yen, P.S.; Amraoui, F.; Rú,a A.V.; Failloux, A.B. *Aedes aegypti* mosquitoes from Guadeloupe (French West Indies) are able to transmit yellow fever virus. *PLOS ONE*. 2018 Sep 1;13(9):e0204710.

715   118.   Morrison, T.E. Reemergence of Chikungunya Virus. *Journal of Virology*. 2014 Oct 15;88(20):11644.

716   119.   Edelman, R.; Tacket, C.O.; Wasserman, S.S.; Bodison, S.A.; Perry, J.G.; Mangiafico, J.A. Phase II safety and immunogenicity study of
717   live chikungunya virus vaccine TSI-GSD-218. *The American journal of tropical medicine and hygiene*. 2000;62(6):681–5.

718   120.   Aikat, B.; Konar, N.; Banerjee, G. Haemorrhagic fever in Calcutta area. *Indian Journal of Medical Research*. 1964;52:660–75.

719   121.   Powers, A.M.; Logue, C.H. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. *The Journal of general*
720   *virology*. 2007 Sep;88(Pt 9):2363–77.

721   122.   Parola, P.; Musso, D. Zika, dengue, chikungunya and yellow fever infections in Europe? – Winter is over, warm days are coming - So
722   hedge your bets. *Travel Medicine and Infectious Disease*. 2020 May;35:101614.

723   123.   Sudeep, A.B.; Parashar, D. Chikungunya: an overview. *Journal of Biosciences*. 2008 33:4. 2008 Nov 1;33(4):443–9.

724   124.   Brighton, S. Chloroquine phosphate treatment of chronic Chikungunya arthritis. An open pilot study - PubMed. *South African Medical*
725   *Journal*. 1984;66(6):217–8.

726   125.   Tan, F.L.; Yin, J.Q. RNAi, a new therapeutic strategy against viral infection. *Cell research*. 2004 Dec;14(6):460–6.

727   126.   Cullen, B.R. Viruses and microRNAs. *Nature Genetics*. 2006 38:6. 2006 May 30;38(6):S25–30.

728   127.   Pavri, K. Presence of chikungunya antibodies in human sera collected from Calcutta and Jamshedpur before 1963. *Indian Journal of*
729   *Medical Research*. 1964;52:698–702.

730   128.   Shah, K.; Gilotra, S.K.; Gibbs, J.C, Rozeboom, L. Laboratory studies of transmission of chikungunya virus by mosquitoes: A preliminary
731   report. *Indian Journal of Medical Research*. 1964;52:703–9.

732   129.   Diallo, M.; Thonnon, J.; Traore-Lamizana, M.; Fontenille, D. Vectors of Chikungunya virus in Senegal: current data and transmission
733   cycles. *The American journal of tropical medicine and hygiene*. 1999;60(2):281–6.

734   130.   Nam, V.S.; Yen, N.T; Holynska, M.; Reid, J.W.; Kay, B.H. National progress in dengue vector control in Vietnam: survey for Mesocyclops
735   (Copepoda), Micronecta (Corixidae), and fish as biological control agents. *Am J Trop Med Hyg*. 2000 Jan;62(1):5–10.

736   131.   Hien, N.T.; Anh, D.D.; Le, N.H.; Yen, N.T.; Phong, T.V.; Nam, V.S.; *et al*. Environmental factors influence the local establishment of
737   *Wolbachia* in *Aedes aegypti* mosquitoes in two small communities in central Vietnam. *Gates Open Res*. 2022 Apr 7;5:147.

738   132.   Sikka, C.V.K.; Popli, R.K.; Galwankar, S.C.; Kelkar, D.; Sawicki, S.G.; *et al*. The emergence of zika virus as a global health security
739   threat: A review and a consensus statement of the INDUSEM Joint working Group (JWG). *Journal of Global Infectious Diseases*. 2016
740   Jan 1;8(1):3.

741   133.   Barrera, R.; Amador, M.; Acevedo, V.; Caban, B; Felix, G.; Mackay, A.J. Use of the CDC Autocidal Gravid Ovitrap to Control and Prevent
742   Outbreaks of Aedes aegypti (Diptera: Culicidae). *Journal of Medical Entomology*. 2014 Jan 1;51(1):145–54.

743   134.   Singh, R.K.; Dhama, K.; Khandia, R.; Munjal, A.; Karthik, K.; Tiwari, R.; *et al*. Prevention and Control Strategies to Counter Zika Virus,
744   a Special Focus on Intervention Approaches against Vector Mosquitoes—Current Updates. *Frontiers in Microbiology*. 2018 Feb
745   8;0(FEB):87.

746   135.   Maciel-de-Freitas, R.; Avendanho, F.C; Santos, R; Sylvestre, G.; Araújo, S.C.; Lima, J.B.P.; *et al* Undesirable Consequences of Insecticide
747   Resistance following *Aedes aegypti* Control Activities Due to a Dengue Outbreak. *PLOS ONE*. 2014 Mar 27;9(3):e92424.

748   136.   Nguyen, T.H.; Nguyen, H L.; Nguyen, T.Y.; Vu, S.N.;Tran, N.D.; Le, T.N.; *et al*. Field evaluation of the establishment potential of w
749   melpop *Wolbachia* in Australia and Vietnam for dengue control. *Parasites & Vectors*. 2015 8:1. 2015 Oct 28;8(1):1–14.

750   137.   Callaway, E. Infected mosquitoes fight Zika. *Nature*. 2016 Oct 26;539(7627):17–8.

751   138.   Walker, T.; Johnson, P.H; Moreira, L.A.; Iturbe-Ormaetxe, I.; Frentiu, F.D.; McMeniman, C.J.; et al. The w Mel *Wolbachia* strain blocks
752   dengue and invades caged *Aedes aegypti* populations. Nature 2011 476:7361. 2011 Aug 24;476(7361):450–3.

753   139.   Vu, S.N.; Nguyen, T.Y.; Tran, V.P.; Truong, U.N.; Le, Q.M.; Le, V.L.; et al. Elimination of dengue by community programs using
754   Mesocyclops (Copepoda) against *Aedes aegypti* in central Vietnam. Am J Trop Med Hyg. 2005 Jan;72(1):67–73.

755   140.   Vythilingam, I.; Sam, J.I.C.; Chan, Y.F.; Khaw, L.T; Sulaiman, W.Y.W. New Paradigms for Virus Detection, Surveillance and Control of
756   Zika Virus Vectors in the Settings of Southeast Asia. *Frontiers in Microbiology*. 2016 Sep 13;0(SEP):1452.

757 141. Alphey, L.; Alphey, N. Five Things to Know about Genetically Modified (GM) Insects for Vector Control. PLOS Pathogens.
758   2014;10(3):e1003909.

759 142. Rattanarithikul, R. A guide to the genera of mosquitoes (diptera: culicidae) of Thailand with illustrated keys, biological notes and
760   preservation and mounting techniques. :70.

761 143. Rattanarithikul, R.; Harrison, B.A.; Harbach, R.E.; Panthusiri ,P.; Coleman, R.E. Illustrated keys to the mosquitoes of Thailand IV.
762   *Anopheles*. 2006;133.

763 144. Green, C.A.; Gass, R.F.; Munstermann, L.E.; Baimai, V. Population-genetic evidence for two species in *Anopheles minimus* in Thailand.
764   *Med Vet Entomol*. 1990 Jan;4(1):25–34.

765 145. Lauri, A.; Mariani, P.O. Potentials and limitations of molecular diagnostic methods in food safety. *Genes Nutr*. 2009 Mar;4(1):1–12.

766 146. Huynh, L.N.; Diarra, A.Z; Pham, Q.L.; Le-Viet, N.; Berenger, J.M.; Ho, V.H.; *et al*. Morphological, molecular and MALDI-TOF MS
767   identification of ticks and tick-associated pathogens in Vietnam. *PLoS Negl Trop Dis*. 2021 Sep;15(9):e0009813.

768 147. Favrot, C. Polymerase chain reaction: advantages and drawbacks. 2015; Available online: https://www.zora.uzh.ch/id/eprint/116536
769   (accessed on 24 Sep 2022).

770 148. Yssouf, A.; Almeras, L; Raoult, D.; Parola, P. Emerging tools for identification of arthropod vectors. *Future Microbiol*. 2016;11(4):549–
771   66.

772 149. Huynh, L.N.; Diarra, A.Z.; Nguyen, H.S.; Tran, L.B.; Do, V.N.; Ly, T.D.A.; *et al*. MALDI-TOF mass spectrometry identification of
773   mosquitoes collected in Vietnam. *Parasit Vectors*. 2022 Jan 28;15:39.

774 150. Dieme, C.; Yssouf, A; Vega-Rúa, A.; Berenger, J.M.; Failloux, A.B.; Raoult, D.; *et al*. Accurate identification of Culicidae at aquatic
775   developmental stages by MALDI-TOF MS profiling. *Parasit Vectors*. 2014 Dec 2;7:544.

776 151. Yssouf, A.; Socolovschi, C.; Flaudrops, C.; Ndiath, M.O.; Sougoufara, S.; Dehecq, J.S.; *et al*. Matrix-Assisted Laser Desorption Ionization
777   - Time of Flight Mass Spectrometry: An Emerging Tool for the Rapid Identification of Mosquito Vectors. *PLoS One*. 2013 Aug 15;8(8).

778 152. Harbach, R.E.; Emma, P.; Bin, Chen, R.K. Butlin. Anopheles (Cellia) minimus theobald (Diptera: Culicidae): neotype designation,
779   characterization, and systematics. *Proceedings of the Entomological Society of Washington*. 2006 Jul 14;108:198–209.

780 153. Niare, S.; Tandina, F.; Davoust, B.; Doumbo, O.; Raoult, D.; Parola, P; *et al*. Accurate identification of *Anopheles gambiae* Giles trophic
781   preferences by MALDI-TOF MS. *Infect Genet Evol*. 2018;63:410–9.

782 154. Shaikevich, E.V.; Vinogradova, E.;, Bouattour, A.; Gouveia, A.A.P. Genetic diversity of *Culex pipiens* mosquitoes in distinct populations
783   from Europe: contribution of Cx. quinquefasciatus in Mediterranean populations. *Parasit Vectors*. 2016 Jan 27;9:47.

784 155. Reuben, R.; Tewari, S.C.; Hiriyan, J.; Akiyama, J. Illustrated keys to species of *Culex* associated with Japanese encephalitis in Southeast
785   Asia (Diptera: Culicidae). *Mosquito Systematics*. 1994;26(2):75–96.

786 156. Batovska, J.; Blacket, M.;, Brown, K.; Lynch, S. Molecular identification of mosquitoes (Diptera: Culicidae) in southeastern Australia.
787   *Ecol Evol*. 2016 May;6(9):3001–11.

788 157. Diarra, A.Z.; Laroche, M; Berger F.; Parola, P. Use of MALDI-TOF MS for the Identification of Chad Mosquitoes and the Origin of Their
789   Blood Meal. *Am J Trop Med Hyg*. 2019;100(1):47–53.

790 158. Bamou, R.; Diarra, A;.Z, Mayi, M.P.A.; Djiappi-Tchamen, B.; Antonio-Nkondjio, C. Parola, P. *Wolbachia* Detection in Field-Collected
791   Mosquitoes from Cameroon. *Insects*. 2021 Dec 17;12(12):1133.

792 159. Laroche, M.; Almeras, L.; Pecchi, E.; Bechah, Y.; Raoult, D; Viola. A; *et al*. MALDI-TOF MS as an innovative tool for detection of
793   *Plasmodium* parasites in *Anopheles* mosquitoes. *Malar J*. 2017 Jan 3;16(1):5.

794 160. Parola, P.; Musso, D.; Raoult, D. *Rickettsia felis*: the next mosquito-borne outbreak? Lancet Infect Dis. 2016 Oct;16(10):1112–3.

795 161. Angelakis, E.; Mediannikov, O; Parola, P.; Raoult, D. *Rickettsia felis*: The Complex Journey of an Emergent Human Pathogen. *Trends
796   Parasitol*. 2016 Jul;32(7):554–64.

797 162. Dieme, C.; Bechah, Y.; Socolovschi, C.; Audoly, G.; Berenger, J.M.; Faye, O.; *et al*. Transmission potential of *Rickettsia felis* infection
798   by *Anopheles gambiae* mosquitoes. *Proc Natl Acad Sci U S A*. 2015 Jun 30;112(26):8088–93.

799   163.   Le-Viet, N.; Le, V.N.; Chung, H.; Phan, D.T.; Phan, Q.D.; Cao, T.V.; *et al*. Prospective case-control analysis of the aetiologies of acute
800   undifferentiated fever in Vietnam. *Emerg Microbes Infect*. 2019 Mar 4;8(1):339–52.
801   164.   Zhang, J.; Lu, G.; Li, J.; Kelly, P.; Li, M.; Wang, J.; *et al*. Molecular Detection of *Rickettsia felis* and *Rickettsia bellii* in Mosquitoes.
802   *Vector-Borne and Zoonotic Diseases*. 2019 Nov 1;19(11):802–9.
803
804

Conclusion and Perspectives

During this thesis, we developed and evaluated for the first time the capacity of MALDI-TOF MS for the identification of field-caught mosquito, tick, and flea species in the Central Highlands of Vietnam. Firstly, we developed a quick and simple protocol to identify mosquitoes stored in silica gel. In our lab, this protocol has been used frequently to identify mosquitoes. It is a quick, low-cost, and easy method for transporting mosquito samples from the field to laboratories, and it could be an alternative to the labor-intensive and expensive freezing preservation method. We have shown this protocol is effective for the rapid and accurate identification of Vietnamese mosquitoes. We have also enriched our in-house MALDI-TOF MS reference database by adding reference spectra of 18 new Vietnamese mosquito species. Particularly, we have deposited all our Raw MS spectra from arthropods included in the reference MS spectra database in a public repository in an attempt to be shared with the entire research community. The MS spectra are available with the DOI number: https://doi.org/10.35081/hwtr-5224. Researchers could download and apply their database system to develop the MS protein spectra database for vector-borne diseases monitoring comprehensively on a global scale.

Secondly, we were the first to use the MALDI-TOF MS for the identification of alcohol-preserved ticks and fleas based on extracted leg and cephalothorax protein spectra, respectively. We have shown that the MALDI-TOF MS is a remarkably useful and reliable robust tool for identifying alcohol-preserved tick and flea species that have undergone different storage periods collected in Vietnam. Additionally, the MALDI-TOF allowed us to identify new tick and flea species that are challenging to identify using morphological and molecular techniques. We identified the DNA of several microorganisms including *Anaplasma platys*, *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Ehrlichia rustica*, *Babesia vogeli*, *Theileria sinensis*, and *Theileria orientalis* in

collected tick species from dogs, cows. We also found *Bartonella clarridgeiae*, *Bartonella rochalimae*, *Bartonella coopersplainsensis*, and *Wolbachia* endosymbiont in fleas collected from cats, dogs, and small wild rodents in Vietnam. We have contributed to improving knowledge of the repertoire of microorganisms associated with ticks and fleas in Vietnam.

Finally, we have written a review on mosquitoes and mosquito-borne diseases in Vietnam. The available data allowed us to see that the checklist of Vietnamese mosquito species is far from being complete as long as further investigations on mosquitoes are carried out and hence mosquito-borne diseases control will continue to be a significant public health concern.

Vector-borne diseases are globally widespread, but they are frequently neglected in developing countries where access to healthcare is limited. Such as the case in Vietnam where *Ehrlichia* spp. infections, the agent of human monocytic ehrlichiosis, was found in ticks collected from wild and domestic animals, have been reported by Kelch WJ (1984) and Parola P *et al*. (2003) and rickettsial infections as a human disease, have been identified in 36,8 % of patients suffering from acute undifferentiated fever (AUF). Despite the potential risks to human health, very little research has been conducted on the molecular identification of ticks, fleas, and mites and their associated microorganisms in Vietnam, with previous studies relying solely on morphological identification. However, there are several drawbacks to both of these methods (morphology and molecular) such as the need for standard taxonomic keys, expert entomology knowledge, time-consuming, exorbitantly priced, and requiring primer-specific targeting for certain species. Recently, MALDI-TOF MS has been proposed as an emerging, alternative and innovative tool for overcoming the drawbacks of the above two methods in medical entomology to identify arthropods.

Previously, I just well-known in entomology and field skills for vector investigation, however, after my Ph.D. thesis, my experience with the MALDI-TOF MS tool in the IHU's VITROME laboratory has enabled me to work on further projects regarding this innovative tool for identification of arthropods and arthropod-borne diseases in Vietnam. In the longer term, I wish in my career as an entomologist to participate in the transfer of my technological skills to Vietnam through training and scientific workshops, as well as conduct a future large-scale study on ectoparasites collected from geographically diverse areas for entomological researchers in future surveys of Vietnamese arthropods and vector control decision-makers.

References

1. Laroche M, Bérenger JM, Delaunay P, Davoust B, Raoult D, Parola P. Medical Entomology: A Reemerging Field of Research to Better Understand Vector-Borne Infectious Diseases. *Clinical Infectious Diseases*. 2017 Aug 15;65(suppl_1):S30–8.

2. Giribet G, Edgecombe GD. The Phylogeny and Evolutionary History of Arthropods. *Current Biology*. 2019 Jun 17;29(12):R592–602.

3. Kampen H. Mechanisms of pathogen transmission by haematophogous arthropods. *Medizinische Welt*. 2007 Jul 1;58:334–41.

4. Levitt AM, Khan AS, Hughes JM. Emerging and re-emerging pathogens and diseases. *Infectious Diseases*. 2010;56–69.

5. Morens DM, Folkers GK, Fauci AS. The challenge of emerging and re-emerging infectious diseases. *Nature*. 2004 Jul;430(6996):242–9.

6. Rattanarithikul R. A guide to the genera of mosquitoes (diptera: culicidae) of Thailand with illustrated keys, biological notes and preservation and mounting techniques. :70.

7. Rattanarithikul R, Harrison BA, Harbach RE, Panthusiri P, Coleman RE. Illustrated keys to the mosquitoes of Thailand IV. Anopheles. 2006;133.

8. Lauri A, Mariani PO. Potentials and limitations of molecular diagnostic methods in food safety. *Genes Nutr*. 2009 Mar;4(1):1–12.

9. Favrot C. Polymerase chain reaction: advantages and drawbacks. 2015 [cited 2020 Sep 24]; Available from: https://www.zora.uzh.ch/id/eprint/116536

10. Yssouf A, Almeras L, Raoult D, Parola P. Emerging tools for identification of arthropod vectors. *Future Microbiol*. 2016;11(4):549–66.

11. Huynh LN, Diarra AZ, Pham QL, Le-Viet N, Berenger JM, Ho VH, et al. Morphological, molecular and MALDI-TOF MS identification of ticks and tick-associated pathogens in Vietnam. *PLoS Negl Trop Dis*. 2021 Sep;15(9):e0009813.

12. Huynh LN, Diarra AZ, Nguyen HS, Tran LB, Do VN, Ly TDA, et al. MALDI-TOF mass spectrometry identification of mosquitoes collected in Vietnam. *Parasit Vectors*. 2022 Jan 28;15:39.

13. Hasnaoui B, Diarra AZ, Berenger JM, Medkour H, Benakhla A, Mediannikov O, et al. Use of the proteomic tool MALDI-TOF MS in termite identification. *Sci Rep*. 2022 Jan 14;12(1):718.

14. Hamlili FZ, Bérenger JM, Diarra AZ, Parola P. Molecular and MALDI-TOF MS identification of swallow bugs Cimex hirundinis (Heteroptera: Cimicidae) and endosymbionts in France. *Parasit Vectors*. 2021 Nov 27;14(1):587.

15. Benyahia H, Ouarti B, Diarra AZ, Boucheikhchoukh M, Meguini MN, Behidji M, et al. Identification of Lice Stored in Alcohol Using MALDI-TOF MS. *J Med Entomol*. 2021 May 15;58(3):1126–33.

16. Sevestre J, Diarra AZ, Laroche M, Almeras L, Parola P. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: an emerging tool for studying the vectors of human infectious diseases. *Future Microbiology*. 2021 Mar 1;16(5):323–40.

17. Le Viet N, Laroche M, Thi Pham HL, Viet NL, Mediannikov O, Raoult D, et al. Use of eschar swabbing for the molecular diagnosis and genotyping of Orientia tsutsugamushi causing scrub typhus in Quang Nam province, Vietnam. *PLoS Negl Trop Dis*. 2017 Feb;11(2):e0005397.

18. Pham K, Hotez PJ. Vietnam: Neglected tropical diseases in an emerging and accelerating economy. *PLoS Negl Trop Dis*. 2022 Feb 17;16(2):e0010140.

19. Do Manh C, Beebe NW, Thi Van VN, Le Quang T, Lein CT, Van Nguyen D, et al. Vectors and malaria transmission in deforested, rural communities in north-central Vietnam. *Malar J*. 2010 Sep 16;9:259.

20. Quang Bui P, Hong Huynh Q, Thanh Tran D, Thanh Le D, Quang Nguyen T, Van Truong H, et al. Pyronaridine-artesunate Efficacy and Safety in Uncomplicated Plasmodium falciparum Malaria in Areas of Artemisinin-resistant Falciparum in Viet Nam (2017-2018). *Clin Infect Dis*. 2020 May 6;70(10):2187–95.

21. Canavati SE, Kelly GC, Vo TH, Tran LK, Ngo TD, Tran DT, et al. Mosquito Net Ownership, Utilization, and Preferences among Mobile and Migrant Populations Sleeping in Forests and Farms in Central Vietnam: A Cross-Sectional Study. *Am J Trop Med Hyg*. 2021 May;104(5):1917–24.

22. Nguyen-Tien T, Probandari A, Ahmad RA. Barriers to Engaging Communities in a Dengue Vector Control Program: An Implementation Research in an Urban Area in Hanoi City, Vietnam. *Am J Trop Med Hyg*. 2019 Apr;100(4):964–73.

23. O'Donnell M, Elston DM. What's Eating You? Human Flea (Pulex irritans). Cutis [Internet]. 2020 Nov 1 [cited 2022 May 4];106(5). Available from: https://www.mdedge.com/dermatology/article/231222/infectious-diseases/whats-eating-you-human-flea-pulex-irritans

24. Stuen S, Granquist EG, Silaghi C. Anaplasma phagocytophilum--a widespread multi-host pathogen with highly adaptive strategies. *Front Cell Infect Microbiol*. 2013;3:31.

25. Parola P, Raoult D. Tropical rickettsioses. *Clinics in Dermatology*. 2006 May 1;24(3):191–200.