MINISTRY OF EDUCATION AND TRAINING MINISTRY OF HEALTH

NATIONAL INSTITUTE OF MALARIOLOGY, PARASITOLOGY AND ENTOMOLOGY

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RESEARCH ON SPECIES COMPOSITION, DENSITY, BEHAVIOR OF *Anopheles*, INFECTION RATE OF *Plasmodium* spp. ON THE PRIMARY MALARIA VECTOR AND DRUD RESISTANCE RELATED MOLECULAR MARKER OF *Plasmodium falciparum* IN FOUR PROVINCE OF THE CENTRAL HIGHLANDS

Major: ENTOMOLOGY

Code: 942 01 06

SUMMARY OF THE DOCTORAL THESIS

HA NOI, 2024

THE THESIS WAS COMPLETED AT

NATIONAL INSTITUTE OF MALARIOLOGY, PARASITOLOGY AND ENTOMOLOGY

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at....., date.....month.....2024

The thesis is available at:

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FOREWORDS

1. Introduction

Over the past decade, Vietnam has been achieving many important achievements in preventing and progressing towards eliminating malaria. However, the Central Highlands region is still facing challenges such as malaria in border groups, migrants, forest goers, and those who sleep in forest that are difficult to control, insecticide-resistant mosquitoes, and drug-resistant malaria parasites. Changes in microclimate factors, natural conditions, the environment, and human impacts have affected the distribution, behavior, species composition, and transmission role of *Anopheles* mosquitoes. Determining *Plasmodium* infection in mosquitoes is important to understand the ecology, geographic distribution, numbers, and behavior of vector species, especially in malaria-endemic geographic areas. Currently, methods for detecting sporozoites include salivary gland dissection, enzyme-linked immunosorbent assay (ELISA), molecular biology such as PCR, real-time PCR, or most recently ddPCR.

The emergence of artemisinin resistance and reduced susceptibility to artemisinin-based combination therapy (ACTs) in *P. falciparum* in the Greater Mekong Subregion has threatened the achievement of malaria control and prevention. The priority is to develop strategies to reduce selective pressure and spread of drug-resistant *P. falciparum* while ensuring that reducing morbidity and mortality [1]. Identification of molecular markers of drug resistance in *P. falciparum* populations is necessary to monitor the distribution and spread of drug resistance and to better understand the evolution and mechanisms of resistance, including artemisinin and ACTs. Furthermore, both theoretically and practically in a malaria-endemic area, if the population of resistant *Plasmodium* spp. in general and *P. falciparum* in particular is carried by the primary and secondary malaria vector populations of *Anopheles* spp. are very dangerous because they spread drug-resistant parasite strains [2], [3], while current anti-malaria drugs are limited in number and new candidate are still in the testing phase.

For these reasons, the thesis titled "Research on species composition, density, behavior of *Anopheles* mosquitoes, infection rate of *Plasmodium* spp. on the primary malaria vector and drug resistance-related molecular marker of *Plasmodium falciparum* in four provinces of the Central Highlands" was carried out with the following goals.

2. Objectives

1. Detemining of species composition, density, and behavior of *Anopheles* spp. mosquitoes and the proportion of *An. minimus* and *An. dirus* infected with *Plasmodium* spp. using molecular techniques in 4 provinces of the Central Highlands, 2019-2022;
2. Analysing the molecular markers related to drug resistance (K_{13}, *plasmepsin* 2, *exonulcease*, *Pfmdr*1, *Pfcrt*) in *P. falciparum* populations in four provinces of the Central Highlands.

3. New contributions and scientific and practical significance of the doctoral thesis

- The study investigated and analyzed to determine the presence of the main malaria vectors *An. minimus* and *An. dirus* in each province in the Central Highlands region - where malaria is complicated and persistent.
- The study showed that the rate of *Plasmodium* spp infection in mosquitoes in four Central Highlands provinces that are in the process of malaria elimination is quite low (<3%).
- The study applied investigation and analysis methods from classical to modern such as

nested-PCR, ddPCR, realtime-qPCR and gene sequencing to clarify molecular aspects, species composition of *Anopheles* spp. and gene mutations related to drug resistance in *Plasmodium* spp. population.

- The connection between the two research contents is meaningful because most of the survey points for insects and drug-resistant parasites are hotspots where the disease is still circulating. This helps policy makers realize that the existence of major vectors infected with drug-resistant *Plasmodium* spp will have the risk of spreading through the mosquito population and the transfer of resistant genes between regions can lead to fail the current achievements.

4. Structure of the doctoral thesis

The thesis has 141 pages including: Introduction: 2 pages; Overview: 33 pages; Research objects and methods: 26 pages; Research results: 43 pages; Discussion: 34 pages; Conclusion: 2 pages; Recommendations: 1 page. The thesis has 22 figures, 29 tables and 139 references.

Chapter 1. LITERATURE REVIEW

1.1. General overview of *Anopheles* mosquitoes

Muỗi *Anopheles* thuộc giới động vật, ngành chân khớp, lớp côn trùng, bộ hai cánh, họ Culicidae, giống *Anopheles*, có mặt khắp ở các vùng ôn đới và nhiệt đới trên thế giới với 721 loài được xếp vào hai phân họ Anophelinae và Culicinae gồm 113 giống [5]. Ở Việt Nam hiện xác định có 64 loài *Anopheles* thuộc 2 phân giống [44], gồm các véc tơ sốt rét chính, véc tơ phụ.

Anopheles mosquitoes belong to the animal kingdom, arthropod phylum, insect class, order Diptera, family Culicidae, genus *Anopheles*, present throughout temperate and tropical regions of the world with 721 species classified into two subfamilies Anophelinae and Culicinae including 113 genera [5]. In Vietnam, there are currently 64 identified species of *Anopheles* belonging to two subgenera [44], including primary malaria vectors and secondary vectors.

***Minimus Complex*:** The *An. minimus* complex comprises three homologous species, namely *An. minimus* and *An. harrisoni* and *An.minimus* E [5]. Research shows that *An. minimus* and *An. harrisoni* are the main vectors in mountainous areas in Eastern countries and are usually at altitudes of 200-900m [15]. *An. minimus* and *An. harrisoni* are found distributed together widely in Northern and Central Vietnam, Southern China, Northern Laos and Western Thailand [5]. In Vietnam, *An. minimus* is the main vector of malaria, distributed in mountainous areas across the country, in mountainous habitats, flowing water, sparse forests, savannas, areas with many streams with sand and gravel, terraced fields [30]. *An. minimus* is nocturnal, peaking from 9pm to 3am, and can be earlier in winter [35]. In the Central Highlands, mosquitoes develop year-round, with the first peak in May and the second peak in September-November; *An. minimus* is active at night and has the highest density from 10pm to 4am [36].

Dirus Complex: *An. dirus* belongs to the *An. leucosphyrus Donitz*, which is the primary vector in Thailand and Southeast Asia. The Dirus complex has 8 members including *An. dirus, An. cracens, An. scanloni, An. baimaii, An. elegans, An. nemophilous, An. takasagoensis* and the recently added species *An. aff. takasagoensis* [5]. Among them, *An. dirus* and *An. baimaii* are vectors that prefer human blood and play a major role in

transmitting malaria through human bites both indoors and outdoors [5]. In Vietnam, *An. dirus* is also the main malaria vector. Research on *An. dirus* by Nguyen Duc Manh (1998) showed that it is distributed in dense forest habitats, multi-layered regenerating forests and dense forests from 20 degrees North latitude and below [39]. *An. dirus* prefers human blood and has the habit of waiting for prey before blood feeding. Mosquitoes are active at night, peaking from midnight to morning, often biting people inside and outside the house, and taking shelter outside the house to drink blood. Mosquitoes develop at their peak during the rainy months.

1.2. Determining the role of *Anopheles* mosquitoes in malaria transmission

A necessary condition for an *Anopheles* species to act as a malaria vector is genetic compatibility between the mosquito and the malaria parasites, which allows the parasite to grow in mosquito [42].

An. minimus s.l. is the main vector in the area where it occurs. In 1990, *An. minimus* s.l. was recorded to be infected with sporozoites throughout the year in Assam (India), except for August-September. The lowest infection rate was in March (0.7%) and the highest in October (8.5%) [58]. A study by Thin OO et al. (2003) [59] on *An. dirus* and its role in malaria transmission in Myanmar reported that *An. dirus* is the main vector of *P. falciparum* infection. In Cambodia and central Vietnam, *An. minimus* was found to be infected with *P. falciparum* and *P. vivax* (Pv210 and Pv247) by ELISA [18]. In the Central Highlands, mosquito dissection also determined that 1.8% of *An. minimus* were infected with malaria parasites. Research by Ho Dinh Trung et al. (2004) [18] determined the rate of *An. dirus* sporozoite infection in Dien Tan commune, Dien Khanh district, Khanh Hoa to be 2.8% and in Binh Thuan to be 1.2%. Research by Nguyen Xuan Quang in National Parks in Kon Tum, Gia Lai and Ea So Nature Reserve in Dak Lak showed that the rate of *An. minimus* parasite infection was 2.19% and that of *An. dirus* was 3.62% [67].

1.3. Application of molecular biology in species identification and determination of malaria parasites in mosquitoes

By using molecular techniques, members of the Minimus complex were recorded in Cambodia [16], southern China, Taiwan, Japan, Laos, Thailand, Vietnam [17], [18]. *An. harrisoni* was recorded in Vietnam, Laos, Thailand, central Myanmar and southern China (up to latitude 32.5N). Ho Dinh Trung and colleagues (2001) used the RFLP-PCR method to distinguish the two species *An. minimus* A and *An. minimus* C in Phu Cuong, Tan Lac-Hoa Binh. Using the PCR molecular identification method, Ngo Thi Huong and colleagues (2004; 2007) identified the Minimus complex in the Central Highlands provinces including two species, *An. minimus* and *An. harrisoni*; the Dirus complex only founded the species *An. dirus* by PCR method.

Identification of *Plasmodium* infection in mosquitoes is a prerequisite for understanding the ecology, geographical distribution, population and behaviour of the vector species. This is especially important in malaria-endemic geographical areas. Up to now, three main methods have been used to identify sporozoite infection, such as salivary gland dissection to identify sporozoites, using ELISA techniques, and PCR methods that are widely used to detect the presence of malaria parasites in mosquitoes and their salivary glands. Claire Y.T Wang et al. (2018) [73] compared two 18S qPCR techniques adapted from digital droplet PCR (ddPCR) and quantified P. falciparum using the Taqman qPCR assay targeting the 18S rRNA gene. High concordance between

ddPCR and qPCR was observed when using both syn18S plasmid DNA standards and oocyst-positive midguts by microscopy. Recent studies of ddPCR for the detection of *Plasmodium* in culture and *Plasmodium* spp.-infected mosquitoes also reported high agreement in parasitemia estimates between ddPCR and qPCR, but sensitivity and precision were improved with ddPCR, especially at low parasitemia cases [74].

1.4. Antimalarial drug resistance in the world and Vietnam

The widespread of CQ-resistant *P. falciparum* strains with failure rates ranging from 70–100% has been reported from various countries in the Greater Mekong Subregion, making this drug no longer recommended. In addition to CQ, resistance to other drugs has also increased, including artemisinin and its derivatives. Therefore, more studies are needed to evaluate the efficacy of new drugs or combination drugs based on the current single drugs used in these countries. In the Mekong Subregion, the delayed parasite clearance in *P. falciparum* and artemisinin resistance are complicated and the rate of D3 asexual persistence is recorded to be increasing. The number of *P. falciparum* cases has decreased, but the persistence of parasitaemia on day 3 after DHA-PPQ treatment has increased from 26% to 45% in parallel with the increase in treatment failures with DHA-PPQ reported from 2008–2013 [78].

1.5. Overview of Plasmodium parasite

P. falciparum has a diverse clinical spectrum and complex pathology, causing malignancy, mortality and multidrug resistance compared to other species. Up to date, 5 species have been recorded to cause disease in humans, including *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*.

1.6. Molecular markers associated with antimalarial drug resistance in *P. falciparum*

1.6.1. Kelch 13 propeller gene (K13)-a molecular marker of artemisinin resistance

The Kelch 13 propeller gene is located in the gene segment from nucleotide 1724848 to nucleotide 1727028 with a length of 2180 nucleotides encoding the Kelch protein with 726 amino acids. The K13 propeller gene was first reported as being associated with artemisinin resistance by Ariey et al. (2014) in Cambodia with 17 mutant alleles, of which 3 mutations appeared at high frequency, including C580Y, R539T and Y493H [89],[91].

Table 1.1. Mutations in the K13 propeller gene according to the WHO classification [92]

Validated		Candidate/Associated	
F446I	P553L	P441L	G538V
N458Y	R561H	G449A	V568G
M476I	C580Y	C469F	P574L
Y493H		A481V	F673I
R539T		P527H	A675V
I543T		N537I	

1.6.2. Molecular marker for piperaquine phosphate resistance

P. falciparum is partially resistant to artemisinin and the molecular marker of artemisinin resistance has now been identified as mutations in the K13 propeller gene. Studies by Benoit Witkowski (2017), Roberto Amato et al. (2017) [100],[101] conducted in Cambodia found that the molecular marker of piperaquine (PPQ) resistance is the amplification of Plasmepsin 2 (PM2) and Plasmepsin 3 (PM3) genes, located on chromosome 14. The plasmepsin gene family consists of 4 genes plasmepsin I, II, III, IV [101], of which

only the PM2-3 gene is associated with increased IC50 in vitro piperaquine test. The wild type of the parasite carries only 1 copy of the PM 2-3 gene; the mutant form carries 2 copies of the PM2-3 gene.

Amato et al. (2017) [101] identified, in addition to the copy number variation of PM 2/3 gene, the exonuclease gene (*exo-E415G* SNP) on chromosome 13 can be presently used as a predictive marker of piperaquine resistance.

1.6.3. Multidrug resistance markers of *P. falciparum* parasites

- *P. falciparum* chloroquin resistance transporter *(Pfcrt)*

P. falciparum chloroquine resistance transporter (Pfcrt) is a 13-exon gene located on a 36kb segment of chromosome 7, which has point mutations associated with CQ resistance from Asia, Africa, and South America [105]. *Pfcrt* is a marker for CQ resistance of *P. falciparum* through mutations in the drug transporter. Mutations in the transporter (crt) activate the process of CQ extrusion from the digestive vacuole, preventing CQ from binding to the heme. Chen (2003) [108] discovered mutations in the *Pfcrt* gene that are resistant to CQ at amino acid positions 72, 74, 75, 76, 97, 144, 160, 220, 271, 326, 356, 371.

- *P. falciparum* multi-drugs resistance (*Pfmdr1*)

Pfmdr1 is a multidrug resistance gene associated with altered susceptibility to multiple drugs. The *Pfmdr1* gene, located on chromosome 5, encodes a 12-transmembrane domain protein called *Pfmdr1* or "Pgh-1". *Pfmdr1* is localized to the digestive vacuole, the site of action of CQ, including quinine. The MQ study data complement the clinical research showing an association between increased *Pfmdr1* copy number and increased risk of failure of MQ monotherapy or combination therapy with ASMQ [113].

Chapter 2. RESEARCH METHODS

2.1. Research method for goal 1

Detemining of species composition, density, and behavior of *Anopheles* spp. mosquitoes and the proportion of *An. minimus* and *An. dirus* infected with *Plasmodium* spp. using molecular techniques in 4 provinces of the Central Highlands, 2019-2022.

2.1.1. Objects, location and time of research

Objects research: Adult *Anopheles* mosquitoes collected at sentinal sites and *Plasmodium* spp. parasites in malaria mosquitoes.

Research conducted from 2019 to 2022.

Select research locations with complex and persistent endemic-malaria areas in the four provinces of Central Highlands Kon Tum, Gia Lai, Dak Lak, and Dak Nong.

2.1.2. Research methods

2.1.2.1. Study design: Descriptive cross-sectional study.

2.1.2.2. Sample size: All Anopheles mosquitoes collected through the methods were identified by external morphology to determine species composition, density and biological behavior of Anopheles mosquitoes at the research sites.

2.1.3. Methods

- Methods on investigation *Anopheles* mosquiotes: indoor light trap (LT-I), outdoor light trap (LT-O), indoor human landing catches (HLC-I), outdoor human landing catches (HLC-O), castle-baited double net trap (CDNT).

- Morphological identification technique of Anopheles mosquitoes.
- DNA extraction.
- PCR method for identifying *An. minimus* s.l. and *An. dirus* s.l.

***PCR method for identifying An. minimus s.l.*:** using primer following the protocol of Hoang Kim Phuc et al (2003).

***PCR method for identifying An. dirus s.l.*:** using primer following the protocol of Ngo Thi Huong et al (2001).

- The presence of *Plasmodium* spp. within the mosquitoes was detected using droplet digital PCR (ddPCR) targeting the 18S rRNA gene region with the primer following protocol of Wampfler et al (2013).

QMAL_fw	TTA GAT TGC TTC CTT CAG TRC CTT ATG
QMAL_rev	TGT TGA GTC AAA TTA AGC CGC AA

2.1.4. Terms and indicators used in objective 1

- Terms: *Anopheles* species composition, *Anopheles* mosquito density, the proportion of *Plasmodium* infection in mosquitoes.
- Indicators: *Anopheles* mosquito density, the proportion of *Plasmodium* infection in mosquitoes.

2.2. Research method for goal 2

Analysing the molecular markers related to drug resistance (K_{13}, *plasmepsin* 2, *exonulcease*, *Pfmdr*1, *Pfcrt*) in *P. falciparum* populations in four provinces of the Central Highlands.

2.2.1. Objects, location and time of research

Objects research: Dry blood spots were collected on Whatman 31 ET chromatography filter paper from the participant who got mono-infection or co-infection with *P. falciparum* in endemic-malaria areas and clinics in four provinces of the Central Highlands.

Research conducted from 2019 to 2022.

Select research locations with complex and persistent endemic-malaria areas in the four provinces of Central Highlands Kon Tum, Gia Lai, Dak Lak, and Dak Nong.

2.2.2. Research methods

2.2.2.1. Study design: Analytical descriptive study.

***2.2.2.2. Samples size*:** Convenience sample collection, collecting all positive blood samples with *P. falciparum* from sentinal sites from 2019 to 2022.

2.2.3. Methods

- Extracted total DNA was used for Nested-PCR techniques to identify four species of malaria parasites; *PCR to capture the K13, Exonuclease, and Pfcrt genes; Sanger sequencing to sequence these gene fragments; and realtime-PCR to identify polymorphisms in the plasmepsin2 and Pfmdr1 genes.*
- PCR-sequencing for determining point mutations in the K_{13} propeller gene [89].

K13_PCR_F	5’-CGGAGTGACCAAATCTGGGA-3’
K13_PCR_R:	5’-GGGAATCTGGTGGTAACAGC-3’
K13_N1_F:	5’-GCCAAGCTGCCATTCATTTG-3’
K13_N1_R:	5’-GCCTTGTTGAAAGAAGCAGA-3’

- PCR-sequencing for determining point mutations in *exonuclease* gene related to piperaquine resistance [101] .

Forward primer	5'- GCA CCT CCT ATC ATC AGA TGA TAC C -3'
Reverse primer	5'- GAA GGT GTT CCT TCC TCT TTT CTT G -3'

- Realtime-PCR to identify polymorphisms in the *plasmepsin2* associated to piperaquine with primer following the protocol [117]

plasmepsinII-1F	5' -ATGGTGATGCAGAAGTTGGA- 3'
plasmepsinII-1R	5' -AACATCCTGCAGTTGTACATTTAAC- 3'
plasmepsinII-probe	5'Fam -CAGGATCTGCTAATTTATGGGTCCCA -BHQ1
βtubulin-1F	5' -TGTGCGCAAGTGATCC- 3'
βtubulin-1R	5' -TTTGTGGACATTCTTCCTC- 3'
β-tubulin-probe	5'HEX-CACATGCCGTTAAATATCTTCCATGTCT-BHQ1

- PCR and Sanger sequencing to sequence these gene fragments of *Pfcrt* gene associated to chloroquin resistance following the protocol of Chen et al (2003) with the primer

D1	5’-TGT GCT CAT GTG TTT AAA CTT-3’
D3	5’-AAA GCT TCG GTG TCG TTC-3’
E3	5’-CTT ATA CAA TTA TCT CGG AGC AGT-3’
F1	5’-GTC ATG TTT GAA AAG CAT ACA GG-3’
E4	5’-CCA AGA ATA AAC ATG CGA AAC C-3’
F2	5’-ATT TCT TAT AGG CTA TGG TAT CC-3’
4A	5’-TAGGAACGACACCGAAG-3’
4B	5’-ATAGTATACTTACCTATATC-3’

- Realtime-PCR to identify polymorphisms in the *Pfmdr1* associated to mefloquine resistance with primer following the protocol of Chavchich M. et al (2010) [118]

MDR1-T1F	TATGCATTTGTGGGAGAATCAG
MDR1-T1R	CTCCTTCGGTTGGATCATAAAG
LDH-T1F	AGGACAATATGGACACTCCGAT
LDHT1R	TTTCAGCTATGGCTTCATCAAA

2.2.4. Indicators used in objective 2

- The proportion of drug-resistant mutation and the proportion of drug-resistant mutation by year.

2.3. Data analysis and processing

- Collected data is recorded in pre-designed forms; Analyzed and processed using Excel software; Applying bioinformatics software such as Geneious R8, genetic data on Genbank to analyze nucleotide sequences of drug resistance genes of *P. falciparum*; Applying R software to draw distribution maps of drug resistance genes.

2.4. Ethical aspects in research

- The thesis outline was approved by the Outline Approval Council and the Biomedical Ethics Council of National Institute of Malariology, Parasitology and Entomology;

- The thesis outline was approved by the Scientific Council and the Biomedical Ethics Council of Institute of Malariology, Parasitology and Entomology Quy Nhon before implementing the research at the Institute;

Chapter 3. RESEARCH RESULTS

3.1. Results on detemining of species composition, density, and behavior of *Anopheles* spp. mosquitoes and the proportion of *An. minimus* and *An. dirus* infected with *Plasmodium* spp. using molecular techniques in 4 provinces of the Central Highlands, 2019-2022

3.1.1. *Anopheles* mosquito species composition at the study sites

The total number of adult mosquitoes collected in the study include 6957 individuals, of which 13 species were collected in Kon Tum, 14 species in Gia Lai, 13 species in Dak Lak and 12 species in Dak Nong.

Table 3.1. Number of Anopheles at study sites by morphological identification

No	Species	Study site				Total
		Kon Tum	Gia Lai	Dak Lak	Dak Nong	
1	*An. (Cell.) dirus* Peyton & Harrison,1979	**89**	**0**	**157**	**141**	**387**
2	*An. (Cell.) minimus* Theobald, 1901	**117**	**199**	**0**	**12**	**328**
3	*An. (Cell.) aconitus* Doenitz, 1902	266	293	284	79	922
4	*An. (Cell.) maculatus* Theobald, 1901	135	345	370	303	1153
5	*An. (Cell.) annularis* Haga 1930	0	16	0	0	16
6	*An. (Ano.) barbirostris* Van der Wulp, 1884	38	95	22	263	418
7	*An. (Ano.) crawfordi* Reid, 1953	14	13	36	140	203
8	*An. (Cell.) jamesi* Theobald,1901	229	523	33	212	997
9	*An. (Ano.) peditaeniatus* Leicester, 1908	25	114	195	296	630
10	*An. (Cell.) philippinensis* Ludlow, 1902	215	163	35	150	563
11	*An. (Ano.) sinensis* Wiedemann, 1828	15	37	141	155	348
12	*An. (Cell.) splendidus* Koidzumi 1920	0	291	48	0	339
13	*An. (Cell.) tessellatus* Theobald, 1901	9	16	23	0	48
14	*An. (Cell.) vagus* Doenitz, 1902	26	12	123	60	221
15	*An. (Cell.) varuna* Iyengar, 1924	13	236	10	125	384
Total		1191	2353	1477	1936	6957

Collecting 715 main malaria vectors *An. dirus* and *An. minimus* in 4 Central Highlands provinces Kon Tum, Gia Lai, Dak Lak and Dak Nong, in which Kon Tum and Dak Nong had both primary vectors, the remaining provinces only detected *An. dirus* or

An. minimus, secondary vectors in mountainous areas including *An. aconitus* and *An. maculatus* were collected, with the largest number being *An. maculatus* with 1153 individuals (16.57%).

3.1.2. Density and biting behavior of Anopheles at research sites

3.1.2.2. Malaria vector density

Table 3.14. Malaria vector density in Kon Tum by all sample collection methods

No	Species	Sample collection methods				
		LT-I c/đ/đ	LT-O c/đ/đ	HLC-I c/ng/đ	HLC-O c/ng/đ	CDNT c/m/đ
1	***An. dirus***	0,25	0	0,38	0,88	2,71
2	***An. minimus***	0,31	0	0	0,44	4,38
3	*An. aconitus*	1,00	0	0,31	0,44	9,92
4	*An. maculatus*	1,19	0	0,13	0,63	4,33
Number of species		**5**	**3**	**4**	**5**	**13**

In Kon Tum, caslte-baited double net trap caught the most species, including 13 species at high density; detected both main vectors *An. dirus* and *An. minimus* by the methods of indoor light trap, human landing catches indoor and outdoor, castle-baited double net trap, but did not catch mosquitoes by the outdoor light trap method; by the human bait method, 5 species of *Anopheles* were caught, 4 species indoors and 5 species outdoors, the total density of biting people outdoors was 2.39 individuals/person/night, much higher than biting people indoors.

Table 3.15. Malaria vector density in Gia Lai by all sample collection methods

No	Species	LT-I c/đ/đ	LT-O c/đ/đ	HLC-I c/ng/đ	HLC-O c/ng/đ	CDNT c/m/đ
1	***An. minimus***	**0,25**	**0**	**0**	**0**	**8,13**
2	*An. aconitus*	0,5	0	0	0	11,87
3	*An. maculatus*	1,82	0,12	0,69	2,81	10,75
Number of species		**8**	**4**	**3**	**6**	**14**

In Gia Lai, similarly in Kon Tum, the most collected mosquitoes were caslte-baited double net trap; only *An. minimus* vectors were detected by the indoor light trap and caslte-baited double net trap methods, not by the outdoor light trap and human bait methods; by the human bait method, 6 Anopheles species were collected, 3 species indoors and 6 species outdoors, the total density of outdoor human bites was 2.81 individuals/person/night, much higher than indoor human bites (0.69 individuals/person/night).

Table 3.16. Malaria vector density in Dak Lak by all sample collection methods

No	Species	LT-I c/đ/đ	LT-O c/đ/đ	HLC-I c/ng/đ	HLC-O c/ng/đ	CDNT c/m/đ
1	***An. dirus***	**2,69**	**0**	**0,31**	**4,38**	**1,63**
2	*An. aconitus*	1,13	0	0,06	0,06	11
3	*An. maculatus*	0,94	0,06	0,5	1,44	13,12
Number of species		**9**	**3**	**4**	**8**	**13**

In Dak Lak, the caslte-baited double net trap method captured the most species, including 13 species at high density; only the vector *An. dirus* was detected by the indoor light trap, human landing catches indoor and outdoor, castle-baited double net trap methods, but not by the outdoor light trap method; by the human bait method, 8 Anopheles

species were captured, 4 species indoors and 8 species outdoors. The total density of human bites outdoors was 5.88 individuals/person/night, much higher than that of human bites indoors.

Table 3.17. Malaria vector density in Dak Nong by all sample collection methods

No	Species	LT-I c/đ/đ	LT-O c/đ/đ	HLC-I c/ng/đ	HLC-O c/ng/đ	CDNT c/m/đ
1	***An. dirus***	**1**	**0**	**0,81**	**1,81**	**3,46**
2	***An. minimus***	**0**	**0**	**0**	**0**	**0,50**
3	*An. aconitus*	0	0	0	0	3,29
4	*An. maculatus*	0,75	0	0	0,25	11,96
Number of species		**5**	**1**	**2**	**3**	**12**

In Dak Nong, the caslte-baited double net trap method caught the most mosquito species, including 12 species, at high density; detected both main vectors *An. dirus* and *An. minimus* by the the indoor light trap, human landing catches indoor and outdoor, castle-baited double net trap methods, but not by the outdoor light trap method; by the human bait method, 3 *Anopheles* species were collected, 1 species indoors and 3 species outdoors, the overall density of biting people outdoors was much higher than biting people indoors.

3.1.2.3. Nocturnal biting activity of malaria vectors

To determine the nocturnal biting activity of malaria vectors, direct human baiting techniques throughout the night indoors and outdoors were carried out at the study sites. The results are presented in the form of graphs.

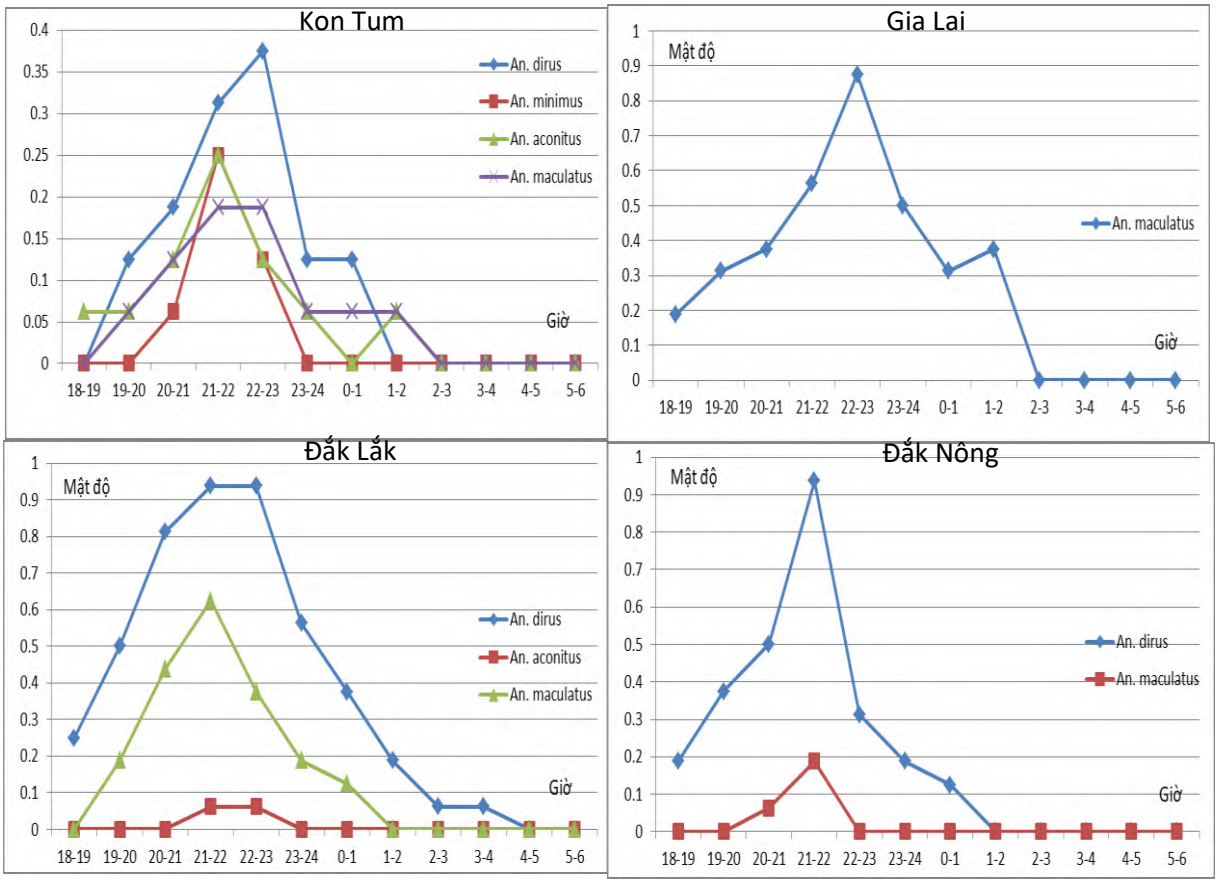

Figure 3.1., 3.2., 3.3., 3.4.: Night-biting time of vectors at study sites

In Kon Tum, vectors started biting humans very early (18-19 hours), the density

increased from 8pm-12pm and then gradually decreased in the morning. The main malaria vector *An. dirus* had a peak biting density from 22pm -23pm with the highest density of 0.38 individuals/hour/person; *An. minimus* had the highest density at 21pm-22pm with an index of 0.25 individuals/hour/person; the secondary vectors *An. aconitus* and *An. maculatus* had peak densities of 0.25 individuals/hour/person and 0.19 individuals/hour/person respectively at 21pm – 23pm.

In Gia Lai, *An. maculatus* was only caught by human baiting, and this secondary vector started biting humans very early (18pm - 19pm), the density increased to 0.88 individuals/hour/person between 22pm - 23pm and then gradually decreased.

In Dak Lak, vectors started biting humans very early (18pm - 19pm), the density increased between 20pm - 24pm and then gradually decreased in the morning. The main malaria vector *An. dirus* had a peak biting density between 21pm - 23pm with the highest density of 0.94 individuals/g/person; the two secondary vectors *An. aconitus* and *An. maculatus* had peak densities of 0.06 individuals/g/person and 0.63 individuals/g/person, respectively, at 21pm -23pm.

In Dak Nong, vectors started biting humans very early (18pm - 19pm), the density increased from 20pm - 23pm and then gradually decreased. The main malaria vector *An. dirus* had a peak biting density from 20pm - 22pm with the highest density of 0.94 individuals/g/person; the secondary vector *An. maculatus* had a peak density of 0.19 individuals/g/person at 21pm - 22pm.

3.1.3. Determining Minimus and Dirus complex by PCR methods

An. minimus s.l and *An. dirus* s.l. were extracted DNA and PCR reactions were performed with target primers to accurately identify the species with predicted theoretical size of about 185 bp for *An. minimus* and 120 bp for *An. dirus*. The results are shown in Figure 3.5-3.7 and Table 3.19

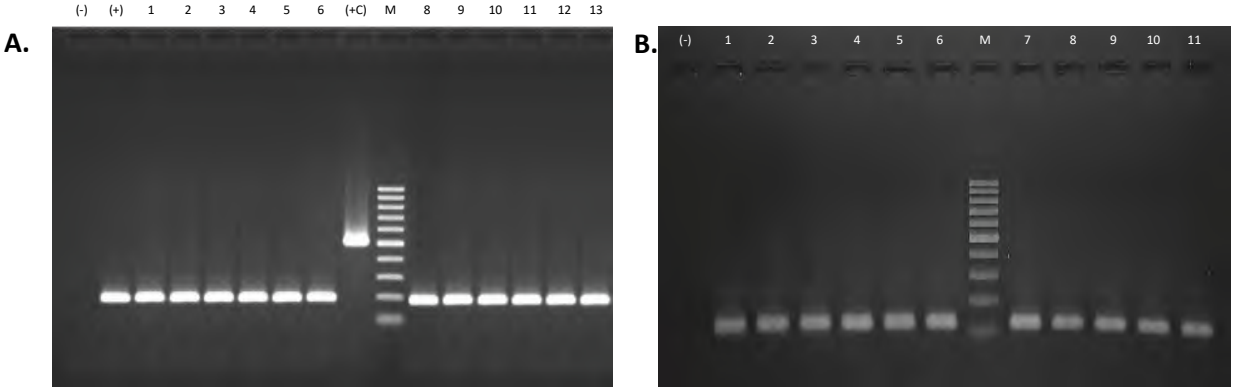

Figure 3.5. và Figure 3.6. The electrophoresis results of PCR product of *Anopheles* species.

Note: A. (-); negative control; (+): possitive control of *An. harrisoni*, 503bp; lane 7: possitive control of *An. minimus*, 185bp; lane 1-13: *An.* minimus, 185bp; M: ladder 100bp. B. (-); negative control; lane 1: positive control of *An. dirus*, 120bp; lane 2-11: *An.* dirus, 120bp; M: ladder 100bp.

Table 3.19. PCR results of Minimus and Dirus complex

No	Study site	Morphological Identification		PCR results		
		An. minimus s.l	***An.dirus s.l***	***An. minimus***	***An. dirus***	**Other *Anopheles***
1	**Kon Tum**	117	89	107	89	2 *An. aconitus* 1 *An. pampanai* 7 *An. varuna*
2	**Gia Lai**	199	-	190	-	1 *An. aconitus* 2 *An. pampanai* 6 *An. varuna*
3	**Đak Lak**	-	157	-	157	-
4	**Đak Nong**	12	141	10	141	2 *An. varuna*
	Total	328	387	307	387	**21**

In genarally, total 715 primary malaria vector, by molecular methods, all 387 *An. dirus* s.l samples obtained in the study gave results as *An. dirus* type A; for 328 *An. minimus* s.l samples, 307/328 samples were classified as *An. minimus* and 21/328 samples gave results as *An. varuna, An. pampanai, An. aconitus*.

3.1.4. Detecting malaria parasites from infected malaria vector *An. minimus* and *An. dirus*

In this study, total 694 of *An. minimus* and *An. dirus* were analyzed by ddPCR technique.

Table 3.21. Number of samples using ddPCR analysis to detect malaria parasites in mosquitoes

Species	Study sites				Total
	Kon Tum	**Gia Lai**	**Dak Lak**	**Dak Nong**	
An. minimus	107	190	0	10	**307**
An. dirus	89	0	157	141	**387**
Total	**196**	**190**	**157**	**151**	**694**

The results of the ddPCR test showed that there were 6 samples positive for the target gene at high concentrations in the reaction, respectively, samples M26 (11 copies/µl), K2 (13.8 copies/µl), M23 (15.2 copies/µl), K1 (19.5 copies/µl), M25 (21.4 copies/µl), KT13 (30.3 copies/µl). From these results, we calculated the target concentration in the initial extracted DNA sample as: 44 copies/µl (M26), 55.2 copies/µl (K2), 60.8 copies/µl (M23), 78 copies/µl (K1), 85.6 copies/µl (M25), 121.2 copies/µl (KT13). In the remaining samples, no positive sample was detected. These are completely negative samples.

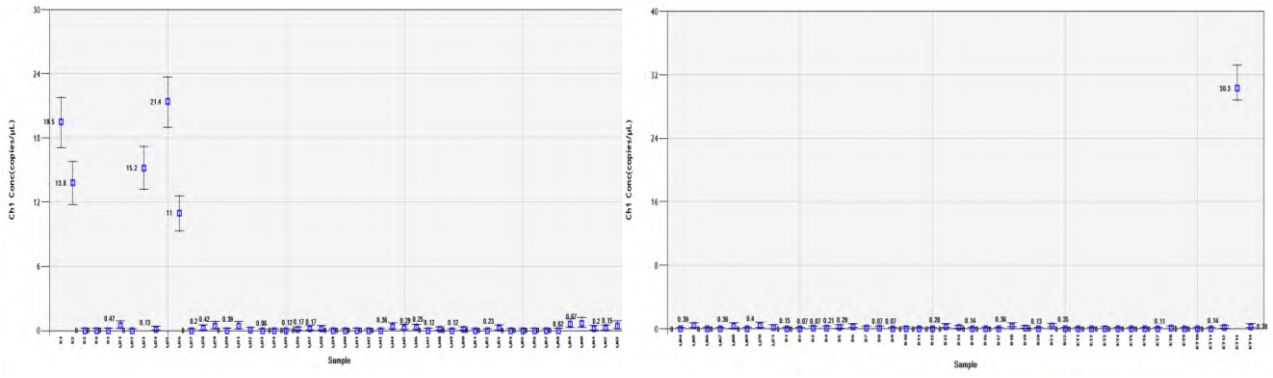

Figure 3.13. Target concentration response curve of the test samples in this study (unit: copies/µl).

Positive samples recorded by ddPCR technique were checked and couted for the number and infection rate of malaria parasites in the main vector as presented in Table 3.22.

Figure 3.22. The proportion of *An. minimus* and *An. dirus* infected with *Plasmodium* spp. by ddPCR

No	Study sites	Primary malaria vector					
		An. minimus			*An. dirus*		
		Number	(+) *Plasmodium*		Number	(+) *Plasmodium*	
			Number	%		Number	%
1	**Kon Tum**	107	3	2,8	89	0	0
2	**Gia Lai**	190	3	1,58	-	-	-
3	**Dak Lak**	-		-	157	0	0
4	**Dak Nong**	10		0	141	0	0
	Total	**307**	**6**	**1,95**	**387**	0	**0**

Note: (+): positive

In Kon Tum, the results of the analysis of the rate of *Plasmodium* spp. infection in 196 samples showed that only 3 *An. minimus* samples were infected with a rate of 2.8%. In Gia Lai, through the analysis of malaria parasite infection using ddPCR technique, 190 *An. minimus* samples recorded 3 cases with the presence of *Plasmodium* spp. accounting for 1.58%. The remaining locations in this study did not detect malaria parasite-infected samples in the main vectors *An. minimus* and An. *dirus*.

3.2. Analysing the molecular markers related to drug resistance K_{13}, *plasmepsin* 2, *exonulcease*, *Pfmdr*1, *Pfcrt*) in *P. falciparum* populations collected from study sites.

3.2.1. Identification of species of *Plasmodium* spp. by using PCR technique

In the study period, dry blood spots were collected on Whatman 31 ET chromatography filter paper from the participant who got mono-infection or co-infection with *P. falciparum*. Extracted total DNA was used for Nested-PCR techniques to confirm the species of malaria parasites. The results are presented in Figure 3.14 and Table 3.23.

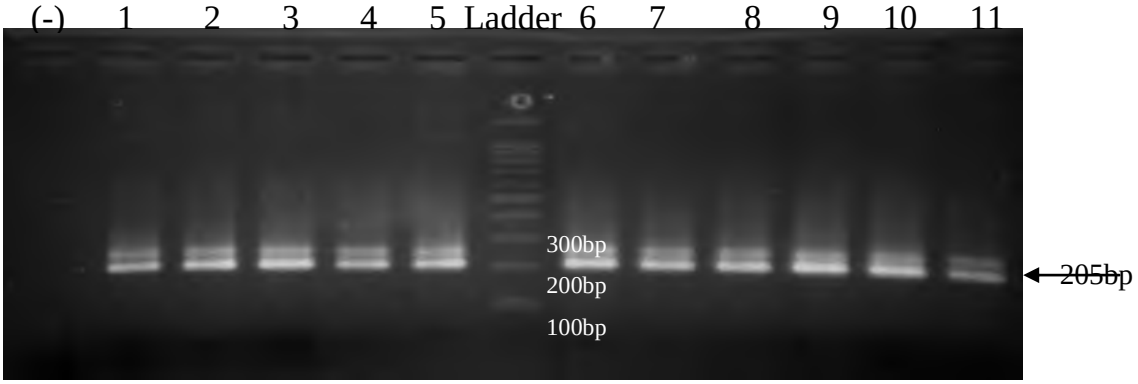

Figure 3.14. Results on identification of *P. falciparum* by PCR technique

Note: (-); negative control; 1: positive control with *P. falciparum*,205 bp; 2-11: *P. falciparum*; M: ladder 100bp.

Table 3.23. Results on identification of *P. falciparum* by PCR technique

Study time	Study sites				Total (n = 562)
	Kon Tum	Gia Lai	Dak Lak	Dak Nong	
2019	23	86	83	44	**236**
2020	-	89	48	82	**219**
2021	-	76	31	-	**107**
Total	**23**	**251**	**162**	**126**	**562**

All of these samples were analysed on PCR technique to confirm the species of *Plasmodium* spp. It was shown that all samples were identified as *P. falciparum* approximatly 205bp.

3.2.2. Results on determining point mutations in the K_{13} propeller gene of *P. falciparum*

A total of 562 *P. falciparum* samples at the study sites, of which 23 samples were in Kon Tum, 251 samples in Gia Lai, 162 samples in Dak Lak and 126 samples in Dak Nong were fully analyzed and detected the presence of 4 mutation positions: R539T, C580Y, I446F and A578S. The number and proportion of K_{13} propeller mutations are summarized and shown in Table 3.24.

Table 3.24. The number and proportion of K_{13} propeller mutations at study site

Study time	Mutants in K_{13} propeller gene	Sentinal sites			
		Kon Tum (N = 23)	Gia Lai (N = 251)	Dak Lak (N = 162)	Dak Nong (N = 126)
2019	***Pfkelch13-C580Y***	30.43% (7/23)	69.77% (60/86)	87.95% (73/83)	79.55% (35/44)
	Pfkelch13-R539T	8.70% (2/23)	0%	0%	0%
2020	***Pfkelch13-C580Y***	-	98.88% (88/89)	77.08% (37/48)	100% (82/82)
	Pfkelch13-R539T	-	0%	2.1% (1/48)	1.3% (1/82)
	Pfkelch13-F446I	-	0%	0%	25.6%(21/82)
2021	***Pfkelch13-C580Y***	-	97.4% (74/76)	100% (31/31)	-
	Pfkelch13-R539T	-	0%	0%	-
	Pfkelch13-A578S	-	1.32% (1/76)	0%	-

Total	***Pfkelch13-C580Y***	30.43%(7/23)	88.45% (222/251)	87.04% (141/162)	87.3% (110/126)
	Pfkelch13-R539T	8.70% (2/23)	0%	0.62% (1/162)	0.79% (1/126)
	Pfkelch13-F446I	-	0%	0%	16.67% (21/126)
	Pfkelch13-A578S		0.40% (1/251)	0%	0%

Thus, in four provinces of the Central Highlands, the presence of the C580Y mutation was recorded at a relatively high rate of 88.45% (222/251), 87.04% (141/162), 87.3% (110/126) respectively in the malaria-endemic areas of Gia Lai, Dak Lak, Dak Nong provinces. Especially, in Kon Tum, the rate of this mutation was not as high as only 30.43% (7/23); In particular, the presence of the new mutation F446I was detected with a rate of 16.67% (21/126) in the sample group of Dak Nong; and a small rate of R539T and A578S mutations.

3.2.3. Results on identification of *Plasmepsin* 2 gene polymorphisms

By using real-time PCR with probe to detect polymorphic variants on the *plasmepsin* 2 gene, all cases had gene amplification reactions, copy number values were recorded and summarized in Table 3.26.

Table 3.26. The number and proportion of *plasmepsin2* copy number variation at study site

Study time	***Plasmepsin2* copy number variation**	**Study sites**			
		Kon Tum (N = 23)	**Gia Lai (N = 251)**	**Dak Lak (N = 162)**	**Dak Nong (N = 126)**
2019	**PM2/3 CNV (>1.5*)***	8.70% (2/23)	69.77% (60/86)	42.17% (35/83)	34.09% (15/44)
2020	**PM2/3 CNV (>1.5)**	-	58.4% (52/89)	79.17% (38/48)	31.71 (26/82)
2021	**PM2/3 CNV (>1.5)**	-	1.32% (1/76)	19.35% (6/31)	-
Total	**PM2/3 CNV (>1.5)**	8.70% (2/23)	45.82% (113/251)	48.77% (79/162)	32.54% (41/126)

Thus, in Kon Tum, Gia Lai, Dak Lak, Dak Nong from 2019 to 2021, the presence of *plasmepsin*2 copy number variation was recorded with the corresponding rates for each province above being 8.7% (2/23), 45.82% (113/251), 48.77% (79/162) and 32.54% (41/126).

3.2.4. Identification of point mutations in the exonuclease gene associated with piperaquine phosphate resistance

The exonuclease gene sequences of 562 *P. falciparum* isolates at the study sites were analyzed using Geneious R8 software and detected the presence of E415G mutation at study sites. The results of the analysis of the number and proportion of E415G mutation are shown in Table 3.27.

Table 3.27. The number and proportion of E415G mutation at study site

Study time	Exo E415G mutation	Study sites			
		Kon Tum (N = 23)	Gia Lai (N = 251)	Dak Lak (N = 162)	Dak Nong (N = 126)
2019	***Exo E415G***	30.43% (7/23)	12.79% (11/86)	49.4% (41/83)	54.55% (24/44)
2020	***Exo E415G***	-	1.12% (1/89)	4.17% (2/48)	59.75% (49/82)
2021	***Exo E415G***	-	3.95% (3/76)	80.6% (25/31)	-
Total	***Exo E415G***	30.43% (7/23)	5.98% (15/251)	41.98% (68/162)	57.94% (73/126)

Tính về tổng thể, tại tỉnh Kon Tum, Gia Lai, Đắk Lắk, Đắk Nông từ năm 2019-2021 ghi nhận có mặt đột biến *exonuclease* E415G với tỷ lệ tương ứng ở từng tỉnh 30,43% (7/23), 5,98% (15/251), 41,98% (68/162) và 57,94% (73/126). Overall, in Kon Tum, Gia Lai, Dak Lak, and Dak Nong provinces from 2019-2021, the presence of exonuclease mutation E415G was recorded with the corresponding rates in each province of 30.43% (7/23), 5.98% (15/251), 41.98% (68/162) and 57.94% (73/126).

3.2.5. Results on identification of point mutations in the *Pfcrt* gene of *P. falciparum*

The *Pfcrt* gene sequences obtained from the sequencing process using Geneious R8 software of 562 *P. falciparum* samples at the research sites were analyzed comparatively, and the presence of mutations *Pfcrt F145I*, *Pfcrt T93S*, *Pfcrt H97Y*, *Pfcrt I218V*, *Pfcrt A220S* and mutations in the region 72-76 on the *Pfcrt* gene segments in the 4 research provinces was detected. The results of the analysis of the number and rate of mutations are shown in Table 3.28.

Table 3.28. The number and proportion of mutations of *Pfcrt* gene at study site

Study time	Mutations of *Pfcrt* gene	Study sites			
		Kon Tum (N = 23)	Gia Lai (N = 251)	Dak Lak (N = 162)	Dak Nong (N = 126)
2019	***Pfcrt (72-76 CVIET)***	95% (22/23)	100%(86/86)	95.2% (79/83)	100% (44/44)
	Pfcrt (72-76 CVIDT)	4.3% (1/23)	0%	2.4% (2/83)	0%
	Pfcrt (72-76 CVVET)	0%	0%	1.2% (1/83)	0%
	Pfcrt (72-76 WGIET)	0%	0%	1.2% (1/83)	0%
	Pfcrt F145I	0%	0%	2.4% (2/83)	18.2% (8/44)
	Pfcrt T93S	13% (3/23)	1.2% (1/86)	18.07%(15/83)	11.36% (5/44)
	Pfcrt H97Y	4.3% (1/23)	0%	6.02% (5/83)	15.9% (7/44)
	Pfcrt I218F	0%	0%	4.82% (4/83)	2.3% (1/44)

	Pfcrt A220S	100%	100%	100%	100%
2020	***Pfcrt (72-76 CVIET)***	-	100%(89/89)	100% (48/48)	100% (82/82)
	Pfcrt F145I	-	0%	4.2% (2/48)	6.1% (5/82)
	Pfcrt T93S	-	5.6% (5/89)	0%	56.1%(46/82)
	Pfcrt I218F	-	0%	0%	6.1% (5/82)
	Pfcrt I218V	-	0%	0%	2.4% (2/82)
	Pfcrt A220S	-	100%	100%	100%
2021	***Pfcrt (72-76 CVIET)***	-	100%(76/76)	100% (31/31)	-
	Pfcrt F145I	-	0%	6.45% (2/31)	-
	Pfcrt T93S	-	1.3% (1/76)	74.19%(23/31)	-
	Pfcrt A220S	-	100%	100%	-

Thus, among 562 *P. falciparum* isolates collected in 4 research provinces, the mutation analysis results on the Pfcrt gene region recorded the presence of 4 halotypes of region 72-76, namely CVIET, CVIDT, CVVET and WGIET, and 5 other mutations including *Pfcrt F145I*, *Pfcrt T93S*, *Pfcrt H97Y*, *Pfcrt I218V* and *PfcrtA220S*. Of which, the CVIET halotype of gene region 72-76 and *PfcrtA220S* dominated with an almost absolute rate, the rate obtained was almost 100% at the research sites except for the sample group in Kon Tum and Dak Lak from 2019-2019.

3.2.6. Results on identification of *Pfmdr1* gene polymorphisms in *P. falciparum*

By using real-time PCR with probe to detect polymorphic variants on *the Pfmdr1* gene, all cases had gene amplification reactions, copy number values were recorded and summarized in table 3.30.

Table 3.30. The number and proportion of *Pfmdr1* copy number variation at study site

Study time	*Pfmdr1* copy number variation	Study sites			
		Kon Tum (N = 23)	**Gia Lai (N = 251)**	**Dak Lak (N = 162)**	**Dak Nong (N = 126)**
2019	***Pfmdr1 CNV (>1,5)***	8.7% (2/23)	5.81% (5/86)	15.66% (13/83)	15.91% (7/44)
2020	***Pfmdr1 CNV (>1,5)***	-	0%	14.58% (7/48)	28.1% (23/82)
2021	***Pfmdr1 CNV (>1,5)***	-	1.32% (1/76)	35.5% (11/31)	-
Tổng số	***Pfmdr1 CNV (>1,5)***	8.7% (2/23)	2.39% (6/251)	19.14% (31/162)	23.81% (30/126)

Thus, in Kon Tum, Gia Lai, Dak Lak, Dak Nong provinces from 2019 to 2021, the presence of *Pfmdr1* copy number variation was recorded with the corresponding rates for each province above being 8.7% (2/23), 2.39% (6/251), 19.14% (31/162) and 23.81% (30/126).

Chapter 4. DISCUSSIONS

4.1. Results of species composition, density, behavior of *Anopheles* mosquitoes, and *Plasmodium* spp. infection rates in *An. minimus* and *An. dirus* using molecular biology techniques in four Central Highlands provinces, 2019–2022

4.1.1. Species composition of *Anopheles* mosquitoes at research sites

A total of 6,957 individual mosquitoes were collected in this study. Regarding species composition, 13 species were found in Kon Tum, 14 in Gia Lai, 13 in Dak Lak, and 12 in Dak Nong. The primary malaria vectors present at these sites included *An. dirus* and *An. minimus*, while secondary vectors included *An. maculatus* and *An. aconitus*. The most abundant species was *An. maculatus*, with 1,153 individuals (16.57%). Morphological identification confirmed the presence of 15 *Anopheles* species, divided into two subgenera: the *Anopheles* Meigen subgenus, with 1,818 individuals across 4 species, and the *Cellia* Theobald subgenus, with 1,902 individuals across 11 species.

The species composition of *Anopheles* mosquitoes in the Central Highlands and the rest of the country shows significant variation between regions, different habitat types, as well as seasonal, host-related, and temporal differences. Le Khanh Thuan et al. (1998) indicated that 48 *Anopheles* species were identified in the Central Highlands, including two primary malaria vectors (*An. dirus* and *An. minimus*) and three secondary vectors (*An. aconitus*, *An. maculatus*, and *An. jeyporiensis*). In a 2011 study on *Anopheles* mosquitoes in the Central Highlands by Nguyen Xuan Quang [46], 14 species were identified in the Dak Ha Nature Reserve in Kon Tum, 17 species in Yok Don National Park, and 15 species in Dak Lak. Another study by Nguyen Xuan Quang et al. (2015) [49] reported 15 species in Gia Lai. Thus, the species composition in this study is lower than in previous studies [67].

These findings suggest that the composition of *Anopheles* species varies across studies and has changed over time. Earlier studies generally reported a higher species diversity than those conducted in the past five years, likely due in part to significant habitat changes, including the gradual reduction of natural forest areas. The presence of primary malaria vectors *An. dirus* and *An. minimus* at the study sites indicates an ongoing risk of malaria transmission and infection for local communities across all four provinces in the Central Highlands.

4.1.2. Some ecological and behavioral characteristics of Anopheles at study sites

4.1.2.1. Malaria vector density

At the research sites in the Central Highlands, both main vectors *An. dirus* and *An. minimus* were detected by the indoor light trap, human landing catches indoor and outdoor, castle-baited double net trap methods. The indoor light trap method caught very few *Anopheles* mosquitoes and almost no malaria vectors. It is possible that when placing light traps indoors, the light source is more concentrated, attracting more mosquitoes to the light, so this method does not accurately reflect the nocturnal activity of mosquitoes indoors or outdoors. This behavior is more accurately assessed by the human bait method.

Research results show that *Anopheles* mosquitoes at research sites in the Central Highlands have a higher habit of biting people outdoors than indoors. Some other studies also showed similar results. Obsomer et al. (2007) when studying the differences in *Anopheles* behavior in Southeast Asia related to forests, hills and factors of *An. dirus* A

and *An. minimus* A showed that the level of mosquito activity outdoors was higher than indoors [20]. Nguyen Xuan Quang et al. (2015) [49], when studying the behavior and disease transmission role of malaria vectors in residential areas and swidden houses in some endemic-malaria areas in the Central Highlands, showed that the main vectors *An. dirus* and *An. minimus* have a higher biting density outdoors.

4.1.2.3. Nocturnal Human-Biting Activity of Malaria Vectors

At research sites across Kon Tum, Gia Lai, Dak Lak, and Dak Nong, malaria vectors show consistent nighttime biting patterns, with activity beginning early (around 18:00 - 19:00) and rising quickly to reach peak density between 22:00 and 23:00. These findings align with previous studies in the Central Highlands, where *An. minimus* was found to bite throughout the night, peaking between 22:00 and 04:00 (Le Khanh Thuan, 1997) [36]. *An. dirus*, in particular, has shown peak biting between 20:00 and midnight.

Earlier research also noted that *An. minimus* bites continuously through the night, although peak times vary with season and location. Typically, *An. minimus* shows peak activity from 21:00 to 03:00, with earlier activity onset during winter months [35]. Compared to studies by Nguyen Xuan Quang (2011, 2019) [67], this study reports similar timing and peak activity patterns. Quang's research in Central Highlands national parks and Khanh Hoa also documented that malaria vectors start biting early in the evening (18:00 - 19:00), with peak densities occurring around 21:00 - 23:00; notably, *An. dirus* tends to reach peak activity earliest.

4.1.3. Identification of the Minimus and Dirus complexes using PCR techniques

In the total of 715 vector samples identified through PCR, all 387 samples of *An. dirus s.l.* were confirmed as *An. dirus* Form A. Among the 328 *An. minimus s.l.* samples, 307 were identified as *An. minimus*, while 21 were classified as *An. varuna*, *An. pampanai*, or *An. aconitus*.

Comparing these findings with previous studies conducted in the Central Highlands, Ngo Thi Huong et al. (2004, 2007) using PCR also identified the Minimus complex in the Central Highlands, specifically comprising two species, *An. minimus* and *An. harrisoni*. For the Dirus complex, only *An. dirus* had been identified. Through PCR analysis, all *An. dirus s.l.* specimens collected at the study sites were confirmed as *An. dirus* Form A. This outcome aligns with findings by Baimai et al. (1987), Ngo Thi Huong et al. (2004) [17], and Truong Van Co et al. (2005) [31].

Thus, for precise species identification, as well as for evaluating behavioral traits and disease transmission roles of individual species within the Minimus complex - especially in areas where both species coexist or where closely related species such as *An. varuna*, *An. pampanai*, *An. aconitus*, and *An. jeyporiensis* are present - the application of molecular biology techniques in entomological investigations is essential and highly valuable.

4.1.4. Identification of the presence of malaria parasites in the bodies of *An. minimus* and *An. dirus* mosquitoes

Analysis of the malaria parasite infection rate in the primary vectors, *An. minimus* and *An. dirus*, in this study showed that only 6 cases of *An. minimus* mosquitoes were found to be positive for malaria parasites, with infection rates of 2.8% in Kon Tum and 1.58% in Gia Lai. Previous studies have reported a malaria parasite infection rate of 2.19% in *An. minimus* and 3.62% in *An. dirus* in national parks in Kon Tum, Gia Lai, and the Ea So Nature Reserve in Dak Lak (Nguyen Xuan Quang, 2012); in the Central

Highlands, mosquito dissection results also revealed 1.8% of *An. minimus* were infected with malaria parasites. In Iakor commune, Gia Lai province, the infection rate of *An. minimus* with *Plasmodium* spp. was 2.58% [36]; Ngo Thi Huong et al. also identified *An. dirus* mosquitoes infected with malaria parasites using the ELISA technique, with an infection rate of 1.22% in Gia Lai and 1.16% in Dak Nong [124]. The malaria parasite infection rate in mosquitoes found in this study is lower than in previous studies, specifically, Nguyen Xuan Quang et al. (2019) used the ELISA technique to determine that the positivity rate for malaria parasites in *An. minimus* at Chu R'Cam reached 11.3%, at Chu Gu 20%, and at Ia Kor 3.85%; the positivity rate for *An. dirus* at Chu Gu was 4.62%. This suggests that the vector species *An. minimus* and *An. dirus* still play a major role in the transmission of malaria in the region, as indicated by the high malaria parasite infection rates in mosquitoes in malaria-endemic areas.

4.2. Molecular markers of drug resistance K_{13}, Plasmepsin 2, Exonuclease, Pfmdr1, Pfcrt in the *Plasmodium falciparum* population at the study site

4.2.1. Identification of point mutations in the K_{13} propeller gene

Results showed that the C580Y mutation was found in *P. falciparum* isolates from all four provinces of the Central Highlands: Kon Tum, Gia Lai, Dak Lak, and Dak Nong, with relatively high rates of 88.45% (222/251), 87.04% (141/162), and 87.3% (110/126) in Gia Lai, Dak Lak, and Dak Nong, respectively. In Kon Tum province, however, the mutation rate was only 30.43% (7/23). The C580Y mutation was also the main mutation detected in the study by Ariey et al. (2014) [89] and the most common mutation in the Greater Mekong Subregion, with a rate of 85%; other mutations with high rates included R539T and Y493H. This is the main mutation found in studies of K_{13} gene mutations in the Greater Mekong Subregion. The C580Y mutation has also been confirmed to be strongly associated with delayed clearance of asexual *P. falciparum* parasites (WHO, 2015).

In addition, analysis of other mutations in the K_{13} gene region also revealed the presence of some previously unreported mutations, such as F446I with a rate of 16.67% (21/126) in the Dak Nong sample group, along with a small proportion of R539T and A578S mutations. These mutations are believed to be associated with delayed "clearance" of asexual *P. falciparum* parasites following artemisinin treatment [131].

4.2.2. Identification of *Plasmepsin* 2 gene polymorphisms

Through real-time PCR analysis using probes to detect polymorphisms in the *plasmepsin* 2 gene, all cases showed gene amplification reactions. Between 2019 and 2021, the presence of copy number variation of *plasmepsin* was recorded with rates of 8.7% (2/23), 45.82% (113/251), 48.77% (79/162), and 32.54% (41/126) in Kon Tum, Gia Lai, Dak Lak, and Dak Nong provinces, respectively. These rates are lower than those reported in other studies. According to Amato et al. (2017), the rate of *plasmepsin*-2 gene polymorphisms increased from 2010 to 2013, reaching 82% in Pursat, 40% in Preah Vihear, and 15% in Ratanakiri [101]. The study by B. Witkowski et al. (2017) also found high polymorphism rates in plasmepsin gene in some provinces during 2014-2015, specifically 91.2% in Pailin, 45.5% in Ratanakiri, 25.4% in Mondulkiri, 91.2% in Siem Reap, and 63% in Stung Treng [100]. Research by Imwong et al. (2020) showed a high rate of plasmepsin-2 gene polymorphisms in some regions of Cambodia, such as Pursat, Pailin, Champasak, and Srisaket [129].

In Vietnam, based on in vivo studies of P. *falciparum* malaria with DHA-PPQ

treatment in multicenter trials, the treatment failure rate was over 10%, with over 10% of patients retaining asexual parasites. Additionally, *P. falciparum* isolates showed both K_{13} gene mutations and an increased *plasmepsin* copy number, indicating resistance to both components of the DHA-PPQ combination, specifically dihydroartemisinin and piperaquine phosphate. These findings provided enough evidence to support a change in the national malaria treatment policy, shifting from DHA-PPQ to pyronaridine tetraphosphate-artesunate (Pyramax®), which has been widely adopted since the end of 2020.

4.2.3. Identification of point mutations in the *exonuclease* gene associated with piperaquine phosphate resistance

In Kon Tum, Gia Lai, Dak Lak, and Dak Nong from 2019 to 2021, the presence of the *exonuclease* E415G mutation was recorded with rates in each province of 30.43% (7/23), 5.98% (15/251), 41.98% (68/162), and 57.94% (73/126), respectively. In Amato et al.'s (2017) study, the prevalence of this mutation was 8% in Ratanakiri, but in Preah Vihear and Pursat, it was significantly higher at 37% and 82%, respectively, in samples from 2013 [101].

4.2.4. Identification of point mutations in the *Pfcrt* gene

Among the 562 *P. falciparum* samples collected, analysis of mutations in the *Pfcrt* gene region identified four halotypes in the 72-76 region: CVIET, CVIDT, CVVET, and WGIET, as well as five other mutations: *Pfcrt F145I*, *Pfcrt T93S*, *Pfcrt H97Y*, *Pfcrt I218V*, and *Pfcrt A220S*. The CVIET halotype in the 72-76 region and Pfcrt*A220S* were predominant, reaching nearly 100% at the study sites, except in Kon Tum and Dak Lak in 2019. This was also reported in Imwong et al.'s (2020) study, which found that the 72-76 locus in the *Pfcrt* gene mutated to the CVIET genotype in most *P. falciparum* isolates in the Greater Mekong Subregion [129].

Clarifying the mechanisms and pathways of antimalarial drug resistance not only provides new directions for malaria research and a deeper understanding of the molecular epidemiology of *P. falciparum* malaria but also contributes to developing new genetic control measures for malaria. This deeper understanding may also enhance the management of other infectious diseases related to drug resistance.

4.2.5. Identification of *Pfmdr1* gene polymorphisms in *Plasmodium falciparum*

During the period from 2019 to 2021, among the 562 samples collected, *Pfmdr1* gene polymorphisms were detected at rates of 8.1% (2/23), 2.39% (6/251), 19.14% (31/162), and 23.81% (30/126) in Kon Tum, Gia Lai, Dak Lak, and Dak Nong provinces, respectively. These rates are similar to findings from other studies in Cambodia. For example, in the study by Amato et al. (2017), the polymorphism rate of the *Pfmdr1* gene was 15% in Preah Vihear, 8% in Pursat, and no samples with *Pfmdr1* gene polymorphisms were recorded in Ratanakiri [101]. The polymorphism rates of the *Pfmdr1* gene in this study in the four Central Highlands provinces of Vietnam are also consistent with rates in some areas of the Greater Mekong Subregion. Specifically, the study by Imwong et al. (2020) reported mutation rates of 13% in Mandalay, 19% in Maesot (Thailand), 4% in southern Laos, and 8% in Stung Treng (northern Cambodia) [129]. These findings align with clinical data showing a correlation between increased *Pfmdr1* copy number and a higher risk of treatment failure in mefloquine monotherapy or artesunate-mefloquine combination therapy from a molecular biology perspective [113]. The World Health Organization (WHO) has also confirmed that an increase in

Pfmdr1 copy number is associated with mefloquine resistance [132].

The status of artemisinin resistance and resistance to combination therapies at the molecular level has spread throughout the Central Highlands region; molecular markers for artemisinin resistance have increased over time. Specifically, in the study by Huynh Hong Quang et al. (2016), the rate of the C580Y mutation in Gia Lai was 36.5%, while in Dak Nong, it was 24.6% [127]. The study by Nguyen Thi Minh Trinh et al. (2016) also identified a C580Y mutation rate of 11.59% (11/96) in Gia Lai [128]. By 2019, the C580Y mutation rate across the four Central Highlands provinces ranged from 30.4% to 87.95%, and by 2020-2021, this rate had risen to 100% in Dak Nong and Dak Lak, indicating that artemisinin resistance is expanding in distribution. Similar results were observed for combination therapies. Polymorphisms in the *plasmepsin* 2 gene and the E415G mutation in the *exonuclease* gene, which are molecular markers for piperaquine phosphate (PPQ) resistance, as well as mutations in the *Pfcrt* gene related to PPQ resistance and *Pfmdr1* gene polymorphisms associated with mefloquine (MQ) and multidrug resistance, have also increased over time.

These results, combined with clinical treatment failure rates and the persistence of D3 asexual forms, indicate a concerning level of drug resistance at the molecular level in the Central Highlands region and across Vietnam. This has been evidenced by the change in malaria treatment policy, shifting from DHA-PPQ to pyronaridine tetraphosphate-artesunate as the primary treatment since late 2020. Currently, widespread drug resistance and reduced sensitivity to several high-efficacy drugs prioritized under the National Malaria Treatment Policy continue to expand. The lack of alternative candidate drugs poses a significant challenge for malaria treatment and elimination efforts. In clinical practice, treatment of *P. falciparum* malaria in Vietnam now involves the use of artesunate-pyronaridine combination therapy (Pyramax®), which still shows high efficacy; however, the rate of D3-positive asexual form persistence remains high, exceeding 30%. Additionally, molecular markers of K_{13} gene mutations, particularly the C580Y phenotype associated with artesunate resistance in the first component of Pyramax in *P. falciparum* populations, indicate partial resistance to artesunate. This underscores the necessity for continuous in vivo drug efficacy monitoring in clinical practice, as well as routine surveillance of drug effectiveness.

These two findings represent top priorities in the malaria elimination strategy from now until 2030, specifically regarding antimalarial drug resistance and vector control for malaria. The current landscape of malaria drug resistance, coupled with the presence of a proportion of mosquito populations infected with malaria parasites at the same research sites over the same timeframe, raises an alarming concern about the spread of drug resistance in the region. This issue has also been explored by recent research groups, which have sought to demonstrate that drug-resistant malaria parasites may be spread through *Anopheles* mosquitoes. The study by Kathrin Witmer et al. (2020) provides clear evidence that clinically confirmed artemisinin-resistant strains, based on K_{13} molecular markers, are capable of transmitting drug resistance to mosquitoes within the scope of antimalarial drug coverage. This finding opens a new direction in current drug resistance research: the potential involvement of mosquitoes in the widespread transmission of drug-resistant malaria parasites and their role in current resistance dissemination [137].

CONCLUSIONS

1. Species composition, density, and behavior of *Anopheles* spp. and the infection rate of *An. minimus* and *An. dirus* with *Plasmodium* spp. in four provinces of the Central Highlands, 2019–2022

- A total of 6,957 adult female mosquitoes were collected across four Central Highlands provinces by using indoor light and outdoor light traps, indoor human and outdoor human bait, and cattle-baited nets. The species distribution was as follows: 13 species in Kon Tum, 14 in Gia Lai, 13 in Dak Lak, and 12 in Dak Nong.
- Of the 6,957 adult female mosquitoes collected, 715 were the primary malaria vectors, including *An. dirus* and *An. minimus*, found across all four Central Highlands provinces: Kon Tum, Gia Lai, Dak Lak, and Dak Nong. Both *An. minimus* and *An. dirus* were present in Kon Tum and Dak Nong, while only *An. minimus* was found in Gia Lai, and *An. dirus* in Dak Lak.
- In the study sites, the majority of mosquitoes were collected by using cattle-baited nets, with higher outdoor human-biting density than indoors. Outdoor light traps captured very few *Anopheles* spp. mosquitoes, none of which were malaria vectors.
- Malaria vectors in the study sites exhibited human-biting behavior throughout the night, with very early activity starting from 18 -19 pm, peaking at 22pm - 23 pm.
- Among the 715 primary malaria vector samples, PCR identification confirmed that all 387 *An. dirus* s.l. specimens collected were *An. dirus* form A. Of the 328 *An. minimus* s.l. samples, 307 were identified as *An. minimus*, and 21 as *An. varuna*, *An. pampanai*, or *An. aconitus*. Of the 307 *An. minimus* samples, 107 were found in Kon Tum, 190 in Gia Lai, and 10 in Dak Nong.
- The infection rates of *Plasmodium* spp. in *An. minimus* mosquitoes were 2.8% in Kon Tum and 1.58% in Gia Lai. No *Plasmodium* spp. infections were recorded at other study sites.

2. Molecular Markers of Drug Resistance (K_{13}, plasmepsin2, Exonuclease E415G, Pfcrt, Pfmdr1) in *Plasmodium falciparum* Populations in Four Central Highlands Provinces

Molecular markers indicating resistance to the antimalarial drugs artemisinin, piperaquine, chloroquine, and mefloquine were detected at the study sites, with the following specific findings:

- K_{13} gene mutations related to artemisinin resistance in *P. falciparum* populations from 2019–2021: The C580Y mutation rate was 30.43% (7/23) in Kon Tum, ranging from 69.77% (60/86) to 98.88% (88/89) in Gia Lai, 77.08% (37/48) to 100% (31/31) in Dak Lak, and from 79.55% (35/44) to 100% (82/82) in Dak Nong. Additionally, a novel F446I mutation genotype, associated with delayed artemisinin clearance, was identified at a rate of 16.67% (21/126) in Dak Nong. The R539T mutation was also detected in 8.7% (2/23) of samples in Kon Tum, 0.62% (1/162) in Dak Lak, and 0.79% (1/126) in Dak Nong.
- *Plasmepsin*2 gene polymorphism associated with piperaquine resistance: The prevalence was 8.7% (2/23) in Kon Tum, 45.82% (113/251) in Gia Lai, 48.77% (79/162) in Dak Lak, and 32.54% (41/126) in Dak Nong.
- E415G mutation in the exonuclease gene related to piperaquine resistance: The mutation rate was 30.43% (7/23) in Kon Tum, 5.98% (15/251) in Gia Lai, 41.98%

(68/162) in Dak Lak, and 57.94% (73/126) in Dak Nong.

- *Pfcrt* gene mutations related to chloroquine resistance: Four halotypes were detected in the 72-76 region -CVIET, CVIDT, CVVET, and WGIET - along with five other mutations: *PfcrtF145I, Pfcrt T93S, Pfcrt H97Y, Pfcrt I218V,* and *PfcrtA220S*. The CVIET haplotype in the 72-76 region accounted for 95-100% of the samples, with *PfcrtA220S* found in 100% of samples across all four provinces.
- *Pfmdr1* gene polymorphisms related to mefloquine resistance: The variant rates in *P. falciparum* populations were 8.7% (2/23) in Kon Tum, 2.39% (6/251) in Gia Lai, 19.14% (31/162) in Dak Lak, and 23.81% (30/126) in Dak Nong.

RECOMMENDATION

- Continue research to identify other mutations as well as expand research sites, especially in areas with severe malaria in the Central Highlands, contributing to supplementing data on molecular drug resistance and some early warning signs of drug resistance. In particularly, it is necessary to focus on research to detect potential molecular markers related to sensitivity-resistance to pyronaridine tetraphossphate in Pyramax tablets, which is the first-line drug currently used in the treatment of uncomplicated *P. falciparum* malaria in Vietnam.
- Studying the role of mosquitoes in the spread of drug-resistant malaria parasites, the existence of major vectors infected with drug-resistant *Plasmodium* spp. will have the risk of spreading through the mosquito population and the transfer of sensitive and resistant genes between regions can fail the current achievements.

THESIS-RELATED PUBLICATIONS

1. Nguyen Thi Minh Trinh, Le Ai Quoc, Le Thi Hanh Dieu, Nguyen Thi Lien Hanh, Nguyen Xuan Quang, Do Van Nguyen, Nguyen Thu Huong, Nguyen Xuan Xa, Huynh Hong Quang (2024). Identification of species composition and malaria parasite (*Plasmodium* spp.) infection rates in mosquitoes using molecular biology techniques in Highlands. *Vietnam Journal of Community Medicine*, ISSN 2354-0613, Vol 65, No. 6, 64-71.
2. Nguyen Thi Minh Trinh, Le Thi Hanh Dieu, Nguyen Thi Lien Hanh, Nguyen Thu Huong, Nguyen Xuan Xa, Huynh Hong Quang (2024). Distribution of drug resistance-related genetic markers of plasmodium falciparum in four provinces of the central highlands (2019-2021). *Vietnam Journal of Community Medicine*, ISSN 2354-0613, Vol 65, No. 6, 79-86.
3. Nguyen Thi Minh Trinh, Le Thi Hanh Dieu, Nguyen Thi Lien Hanh, Nguyen Thu Huong, Nguyen Xuan Xa, Huynh Hong Quang (2024). Molecular dynamics of artemisinin resistance in *Plasmodium falciparum* in several provinces of central highghlands 2016-2021. *Vietnam Journal of Community Medicine*, ISSN 2354-0613, Vol 65, No. 6, 72-78.