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GENETIC VARIATIONS IN DRUG RESISTANCE GENES AND THEIR ASSOCIATION WITH DIHYDROARTEMISININ - PIPERAQUINE RESISTANCE IN *Plasmodium falciparum* STRAINS IN MALARIA-ENDEMIC AREAS.

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INTRODUCTION

Malaria is a severe infectious disease with high prevalence in tropical and subtropical regions. Among the causative agents, *Plasmodium falciparum* is responsible for the highest mortality rates. The World Health Organization (WHO) recommends artemisinin-based combination therapy (ACT), comprising dihydroartemisinin (DHA) and piperaquine (PPQ), as the first-line treatment for uncomplicated *P. falciparum* malaria in Southeast Asian countries, including Cambodia, Thailand, Laos, and Vietnam. However, the emergence of drug-resistant *P. falciparum* strains has compromised the efficacy of this therapeutic regimen in several regions.

To address these challenges, evaluating therapeutic efficacy and monitoring drug resistance are essential. Studies on the efficacy of DHA-PPQ provide critical data for updating treatment guidelines and preserving the effectiveness of current therapies, thereby contributing to the reduction of malaria morbidity and mortality.

Molecular markers play a crucial role in providing insights into the presence and spread of drug resistance, enabling the adjustment of treatment regimens. Genes such as *pfK13 (Plasmodium falciparum Kelch 13*), *pfpm2 (Plasmodium falciparum plasmepsin2),* and *pfEXO (Plasmodium falciparum exonuclease*) are utilized to monitor resistance in *P. falciparum.*

The *pfK13* gene encodes the Kelch protein, which is associated with artemisinin resistance. The WHO has identified several mutations, including C580Y, R539T, Y493H, I543T, and N458Y, as molecular markers of artemisinin resistance. The *pfpm2* and *pfEXO* genes are linked to piperaquine resistance, with specific mutations and copy number variations reducing the dug's therapeutic efficacy.

To further investigate the issue of drug resistance, I undertook the dissertation entitled *" **Genetic variations in drug resistance genes and their association with Dihydroartemisinin -Piperaquine resistance in Plasmodium falciparum strains in malaria-endemic areas "***

Rearch Objectives:

1- To investigate genetic variations and the mutation frequencies of key drug resistance genes in *P. falciparum* malaria parasites.

2- To assess the therapeutic efficacy of dihydroartemisinin-piperaquine (DHA-PPQ) in the management of uncomplicated malaria caused by P. *falciparum.*

3- To analyze factors influencing the efficacy of DHA-PPQ in the treatment of uncomplicated *P. falciparum malaria.*

Chapter 1. LITERATURE REVIEW

1.1. Malaria

Malaria is a parasitic infectious disease caused primarily by five species of the Plasmodium parasite: *Plasmodium falciparum, P. vivax, P. ovale, P. malariae, and P. knowlesi.* Among these, P. *falciparum* is the most deadly, responsible for the majority of severe cases and fatalities worldwide. The disease is transmitted to humans through the bites of infected female *Anopheles* mosquitoes, which thrive in tropical and subtropical climates.

Malaria presents with a spectrum of clinical manifestations that vary based on the *Plasmodium* species involved, the patient's age, and their level of immunity. In cases of uncomplicated malaria, individuals typically experience cyclical fevers accompanied by chills and sweats, which correspond to the synchronized rupture of infected red blood cells. Other common symptoms include severe headaches, muscle pain, fatigue, and a general sense of malaise as the body responds to the infection. If left untreated, malaria can progress to severe forms, which are life-threatening and require immediate medical attention. Severe malaria may lead to cerebral malaria, characterized by impaired consciousness, seizures, and coma due to the sequestration of parasites in the brain's microvasculature.

The cornerstone of current *P. falciparum* malaria treatment is ACT. One of the preferred regimens for uncomplicated malaria is DHA-PPQ, which combines two agents to enhance therapeutic efficacy and reduce the risk of parasite resistance.

However, emerging resistance to DHA-PPQ in certain endemic areas poses a significant challenge, leading to treatment failures, prolonged illness, and an elevated risk of severe complications. Consequently, monitoring therapeutic effectiveness and conducting molecular surveillance of resistance are critical for controlling and preventing malaria, particularly in *P. falciparum*–endemic regions.

1.2. Dihydroartemisinin–Piperaquine in the Treatment of Uncomplicated *P. falciparum* Malaria

Dihydroartemisinin–piperaquine (DHA–PPQ) is recognized as one of the most effective ACT for managing uncomplicated *P. falciparum* malaria. DHA is the biologically active derivative of artemisinin, a compound initially extracted from the plant Artemisia annua. Its antimalarial action is mediated by reactive oxygen species and free radicals that disrupt parasite cell membranes, ultimately leading to parasite death. Piperaquine, on the other hand, is a bisquinoline compound with a longer half-life, enabling sustained drug levels in the bloodstream. By combining these two agents, DHA–PPQ provides both rapid parasite clearance (from DHA) and extended protective action (from piperaquine), resulting in high efficacy and a lower likelihood of recrudescence.

Clinical trials and large-scale field studies have consistently shown that DHA–PPQ achieves high cure rates when administered according to recommended dosage and duration guidelines. The regimen is particularly valued for its convenient once-daily dosing schedule and generally favorable safety profile. In regions with confirmed susceptibility to DHA–PPQ, this combination therapy significantly reduces the risk of treatment failure, shortens illness

duration, and helps curb malaria transmission by expediting parasite clearance from the human host.

Despite these advantages, resistance to artemisinin and its partner drugs has emerged in some endemic areas, posing a threat to the long-term utility of DHA–PPQ. Continuous monitoring through *in vivo* Therapeutic Efficacy Studies (TES) and *in vitro* or molecular assays is therefore critical for detecting early shifts in drug sensitivity. These efforts allow public health authorities and clinicians to adapt treatment strategies promptly—such as rotating partner drugs or introducing novel combination therapies—to preserve the efficacy of DHA–PPQ and sustain progress in malaria control.

1.3. Antimalarial drug resistance and Mutations in resistance genes.

Antimalarial drug resistance, particularly in *P. falciparum,* is a growing threat to global malaria control, driven by mutations in key resistance genes such as *Pfkelch13 (pfk13*), plasmepsin 2 (pfpm2), and *PfEXO.* Mutations in the *pfk13* gene, especially in the propeller domain, are the primary markers of artemisinin resistance. Common mutations like C580Y, R539T, and F446I are associated with delayed parasite clearance and are most prevalent in the Greater Mekong Subregion (GMS). Although artemisinin resistance alone does not typically lead to treatment failure, its combination with partner drug resistance poses a significant challenge. Piperaquine resistance, for instance, is linked to amplification of the *pfpm2* gene, which increases the parasite's ability to counteract the drug's effects. This resistance has been observed in regions of the GMS, where DHA-PPQ treatment failures are common. Additionally, the E415G

mutation in the *PfEXO* gene has been associated with multidrug-resistant profiles, though its role in resistance mechanisms requires further study.

The spread of these mutations has serious clinical and epidemiological implications. Treatment failures due to a combination of *Pfk13* mutations and partner drug resistance have been documented, particularly with DHA-PPQ, necessitating alternative therapies in affected regions. Molecular surveillance plays a critical role in tracking these mutations, detecting emerging resistance, and informing treatment guidelines. In Africa, the detection of *pfk13* mutations raises concerns about the potential spread of resistance across the continent, threatening malaria control efforts. To combat resistance, advanced genomic tools like next-generation sequencing are being employed to improve detection and monitoring. Integrated approaches combining clinical, parasitological, and molecular data are essential to guide policy decisions, ensure the continued efficacy of ACTs, and sustain progress toward malaria elimination.

Chapter 2. MATERIAL AND METHODS

2.1. Study Subjects

Objective 1: Dried blood spot samples on Whatman filter paper were collected from 421 patients diagnosed with uncomplicated malaria due to mono-infection with *P. falciparum*, as confirmed by Giemsa-stained blood smear microscopy. The samples were obtained between August 2018 and May 2019 from four provinces in Vietnam: Đắk Nông, Gia Lai, Đắk Lắk, and Bình Phước. All samples satisfied the following criteria:

Inclusion Criteria:

- Mono-infection with *P. falciparum.*
- No restriction on age or gender.
- Written consent provided by the patient or their legal guardian/parent to participate in the study.

Exclusion Criteria:

- Mixed-species infections or mono-infections with species other than *P. falciparum.*
- Declined to provide consent for participation in the study.

Objective 2: The medical records of patients who underwent an evaluation of the therapeutic efficacy of the DHA-PPQ regimen in the treatment of uncomplicated malaria caused by *P. falciparum* was reviewed. The study period spanned from August 2018 to May 2019.

Inclusion Criteria:

Medical Records of Patients Treated with DHA-PPQ and Monitored Post-Treatment Following the WHO (2009) Standard Protocol. Eligible patients fulfilled the following criteria:

- Comprehensive administrative information, including full name, age, gender, medical record ID, and address.

- Detailed clinical information, including initial symptoms, confirmed diagnosis, and disease progression.
- Relevant paraclinical data, such as laboratory results, body temperature, and parasite density.
- Complete follow-up data before, during, and after treatment, in line with the WHO (2009) guidelines.

Exclusion Criteria:

- Medical records that are torn, missing pages, illegible, or contain inaccurate information.
- Missing critical information such as diagnosis, laboratory results, or follow-up data.
- Inappropriate diagnosis: Records do not confirm the presence of *P. falciparum* or indicate co-infection with other *Plasmodium* species.
- Patients diagnosed with complicated malaria or severe comorbidities such as liver failure, kidney failure, or HIV/AIDS.

Objective 3: The medical records of 63 patients diagnosed with uncomplicated malaria caused by *P. falciparum*, treated with DHA-PPQ, along with genetic mutation data for the *pfK13, pfpm2*, and *pfEXO* genes in *P. falciparum* parasites.

2.2. Ethics

The study was approved by the Ethics Committee in Research of the Military Medical Academy under Certificate No. 1690/GCN-HVQY dated June 4, 2018. All participants were informed about the benefits, objectives of the study, and the procedures employed for data collection.

2.3. Study duration, location

- Location: Institute of Medical and Pharmaceutical Research, Vietnam Military Medical University.
- Time duration: From Auguts 2018 to March 2020

2.4. Chemicals, Equipment, tools

The chemicals, machinery, and equipment used for this study were provided by the Institute of Medical and Pharmaceutical Research, Vietnam Military Medical University

2.5. Research method and study design

2.5.1. Study design

- A descriptive study combined with analytical components to investigate genetic variations, therapeutic efficacy, and associated factors in *P. falciparum* malaria treatment.
- The research involved molecular analysis of mutations in the *pfK13, pfpm2, and pfEXO* genes, as well as clinical and follow-up data from patients treated with the DHA-PPQ regimen.

2.5.2. Sample size

The sample size was calculated using the following formula:

$$n=Z^2_{1-\alpha/2}\frac{1-p}{\varepsilon^2 p}$$

To account for all three genes, the highest required sample size (n=326) was adjusted for a 10% data loss, resulting in a final requirement of 356 samples. The study exceeded this requirement by collecting data from 421 patients with uncomplicated *P. falciparum* malaria, ensuring robust analysis of genetic variations and mutation frequencies in drug resistance genes.

Objective 2: The sample size for the retrospective study was determined using the WHO (2009) reference table. Based on a clinical failure rate of 10% for DHA-PPQ treatment, a 95%

confidence level, and a precision of 10%, the base sample size was calculated to be 49 patients. To account for a potential 10% loss to follow-up or withdrawal, the required sample size was adjusted to 57 patients. To ensure sufficient data for analysis, the study selected 63 patient records, exceeding the calculated requirement and providing robust data for the evaluation.

2.6. Methodological Approaches

- Parasite Density Counting: Parasite density was determined using Giemsa-stained blood smear microscopy to quantify *P. falciparum infections.*
- Genomic DNA Extraction
- DNA Quantification and Purity Assessment
- Molecular Analysis of Candidate Genes:
    - *pfK13* mutations: Identified using PCR followed by gene sequencing to detect key resistance-associated mutations.
    - *pfpm2* copy number variations: Amplified and quantified using Real-Time PCR (qPCR).
    - *pfEXO* mutations: Detected through ARMS-PCR (Amplification Refractory Mutation System PCR).
- The therapeutic efficacy of DHA-PPQ was assessed by analyzing archived medical records of patients who participated in clinical trials evaluating the treatment regimen.

2.7. Data analysis

Statistical analyses were conducted using R software (version 4.2.1), with all significance levels set at $p<0.05$

Chapter 3. RESULTS

3.1. Genetic variations and the mutation frequencies of key drug resistance genes in *P. falciparum* malaria parasites.

3.1.1. Prevelance of *pfK13* mutation

Table 3. 1: Distribution of *pfK13* Mutations Across Study Sites

	pfK13; n(%)			
Study site	C580Y mutation	Wild type	**Total**	**P**[1]
Binh Phuoc	37,0 (86,0)	6,0 (14,0)	43,0 (100)	0,042
Dac Lak	89,0 (95,7)	4,0 (4,3)	93,0 (100)	
Dak Nong	46,0 (83,6)	9,0 (16,4)	55,0 (100)	
Gia Lai	184,0 (92,5)	15,0 (7,5)	199,0 (100)	
Total	356,0 (91,3)	34,0 (8,7)	390,0 (100)	

[1]*Fisher's exact test*

Among the initial 421 *P. falciparum* samples collected, 390 samples were successfully sequenced. The overall frequency of the C580Y mutation in the *pfK13* gene among the sequenced samples was 91.3% (356/390), while the wild-type allele was detected in 8.7% (34/390). Mutation frequencies varied across the study sites, with significant differences observed. In Bình Phước, the C580Y mutation was present in 86.0% of cases (37/43), with a statistically significant result (P=0.042). In Đắk Lắk, the frequency was 95.7% (89/93), while in Đắk Nông and Gia Lai, the frequencies were 83.6% (46/55) and 92.5% (184/199), respectively.

3.2.2. Prevalance of pfpm2 copy number variation

Table 3.2: Distribution of *pfpm2* Copy Numbers Across Study Sites

pfpm2; n(%)				
Study site	Single copy	Multiple copies	**Total**	**P**[1]
Binh Phuoc	21 (55,3)	17 (44,7)	38 (100)	0,2
Dak Lak	52 (57,1)	39 (42,9)	91 (100)	
Dak Nong	9 (47,4)	10 (52,6)	19 (100)	
Gia Lai	92 (44,4)	115 (55,6)	207 (100)	
Total	**174(49,0)**	**181(51,0)**	**355(100)**	

[1]Pearson's Chi-squared test

Across the study sites, the frequency of multiple copies of the *pfpm2* gene varied. In Bình Phước, 44.7% (17/38) of samples showed multiple copies, while Đắk Lắk reported 42.9% (39/91). In Đắk Nông, 52.6% (10/19) of samples had multiple copies, and Gia Lai had the highest frequency at 55.6% (115/207). Overall, 51.0% (181/355) of samples across all sites contained multiple copies of the *pfpm2* gene. However, Pearson's Chi-squared test revealed no statistically significant difference in the distribution of multiple copies among the study sites P=0.2).

3.2.3. Prevalence of E415G mutation

Table 3: Distribution of *pfEXO* E415G Mutation Across Study Sites

pfEXO; *n(%)*				
Study site	E415G Mutation	Wild type	**Total**	**P**[1]
Binh Phuoc	27 (84,4)	5 (15,6)	32 (100)	0,014
Dak Lak	79 (85,9)	13 (14,1)	92 (100)	
Dak Nong	44 (78,6)	12 (21,4)	56 (100)	
Gia Lai	149 (70,0)	64 (30,0)	213 (100)	
Total	299 (76,1)	94 (23,9)	393 (100)	

[1]Pearson's Chi-squared test

The frequency of the E415G mutation in the *pfEXO* gene varied across the study sites. In Bình Phước, the mutation was detected in

84.4% of samples (27/32), while in Đắk Lắk, the frequency was slightly higher at 85.9% (79/92). In Đắk Nông, the mutation was present in 78.6% of cases (44/56), and Gia Lai recorded the lowest frequency at 70.0% (149/213). Overall, the E415G mutation was observed in 76.1% (299/393) of all samples. Pearson's Chi-squared test revealed a statistically significant difference in the distribution of the mutation across the study sites (P=0.014).

3.2.4. Prevalence of combine genotype

Table 4: Distribution of combine genotype across study site

	Study site n(%)					
Genotype combination	Binh Phuoc	Dak Lak	Dak Nong	Gia Lai	Total	**P**[1]
C580Y+ *pfpm2*	13/37 (35,1)	33/83 (39,8)	6/17 (35,3)	98/193 (50,8)	150/330 (45,5)	0,13
C580Y+ E415G	25/31 (80,6)	69/84 (82,1)	35/52 (67,3)	121/199 (60,8)	250/366 (68,3)	0,002
E415G/*pfpm2*	9/27 (33,3)	36/83 (43,4)	6/16 (37,5)	74/206 (35,9)	125/332 (37,7)	0,7
C580Y+ E415G+ *pfpm2*	9/26 (34,6)	30/75 (40,0)	5/15 (33,3)	59/193 (30,6)	103/309 (33,3)	0,5

[1]Fisher's exact test

The table presents the distribution of combined drug resistance genotypes involving C580Y, *pfpm2,* and E415G mutations across study sites in Vietnam. Overall, the frequency of parasites carrying complex genotypes, such as C580Y + *pfpm2*, C580Y + E415G, and C580Y + E415G + *pfpm2,* was relatively high across study sites, indicating a significant prevalence of multidrug resistance markers in *P. falciparum* populations in Vietnam.

3.3. The therapeutic efficacy of dihydroartemisinin-piperaquine in the management of uncomplicated malaria caused by *P. falciparum*

Table 5. Treatment out come of DHA-PPQ post 42 day follow –up

Treatment out come		Binh Phuoc n, %	Dak Nong n, %	Total n, %	*p*-Value *
Completed 42-Day Follow-Up	Early Treatment Failure (ETF)	0 (0)	0 (0)	0 (0)	0,042
	Late Clinical Failure (LCF)	14 (31,8)	1 (5,3)	15 (23,8)	
	Late Parasitological Failure (LPF)	3 (6,8)	3 (15,8)	6 (9,5)	
	Adequate Clinical and Parasitological Response (ACPR)	21 (47,7)	11 (57,9)	32 (50,8)	
LOSS/WTH*	LOSS (Loss to follow-up)	3 (6,8)	4 (21,1)	7 (11,1)	
	withdrawal (WTH)	3 (6,8)	0 (0)	3 (4,8)	

The therapeutic efficacy of DHA-PPQ was assessed based on treatment outcomes among patients who completed the 42-day follow-up. The results showed no cases of ETF in either Bình Phước or Đắk Nông. LCF was observed in 31.8% (14/44) of patients in Bình Phước and 5.3% (1/19) in Đắk Nông. LPF was reported in 6.8% (3/44) of patients in Bình Phước and 15.8% (3/19) in Đắk Nông. ACPR, indicating successful treatment, was achieved in 47.7% (21/44) of patients in Bình Phước and 57.9% (11/19) in Đắk Nông, with an overall ACPR rate of 50.8% (32/63). These findings highlight variations in DHA-PPQ efficacy across different regions, emphasizing the need for localized monitoring and tailored interventions.

3.4. Analysis of Factors Affecting DHA-PPQ Efficacy in Uncomplicated Falciparum Malaria

Table 3.6. Logistic Regression Analysis of Genetic Factors Associated with DHA-PPQ Treatment Outcomes

Factors		Treatment outcome		OR 95% CI	P
		Failure	Response		
pfK13/pfEXO	Absent	2	9	-	-
	Present	13	0	4,5 (0,93-33,45)	0,085
pfK13/pfpm2	Absent	10	18	-	-
	Present	11	4	4,95 (1,32-21,88)	**0,023**
pfpm2/pfEXO	Absent	7	14	-	-
	Present	8	3	5,33 (1,15-30,81)	**0,041**
pfK13/pfpm2/pfEXO	Absent	7	13	-	-
	Present	8	3	4,95 (1,06-28,75)	**0,05**

P: Wald test

The logistic regression analysis highlights the significant role of genetic mutations in *pfK13, pfpm2, and pfEXO* in DHA-PPQ treatment outcomes. The presence of *pfK13* and *pfEXO* mutations increased the odds of treatment failure (OR = 4.5, 95% CI: 0.93–33.45), though this result was not statistically significant (p=0.085). In contrast, the combination of *pfK13* and *pfpm2* showed a

significant association with treatment failure (OR = 4.95, 95% CI: 1.32–21.88,

p=0.023), indicating that parasites carrying both mutations are nearly five times more likely to fail treatment. Similarly, the combination of pfpm2 and pfEXO mutations demonstrated a strong and significant association with treatment failure (OR = 5.33, 95% CI: 1.15–30.81, p=0.041). Furthermore, parasites with all three mutations (*pfK13, pfpm2, and pfEXO)* exhibited an OR of 4.95 (95% CI: 1.06–28.75, p=0.05), suggesting a significant but borderline association with treatment failure.

After adjusting for factors such as age, BMI, and malaria parasite density, the OR for treatment failure associated with mutations in *pfpm2/pfEXO* increased to 7.78, with a statistically significant difference ($p<0.05$). Patients harboring parasites with mutations in *pfpm2/pfEXO* were found to have a 5.33-fold higher risk of treatment failure compared to those without these mutations (95%CI:1.15–30.81, $p<0.05$). Following adjustment for age, BMI, and parasite density, the OR further increased to 8.12, reinforcing the strong association between these mutations and treatment failure.

Similarly, patients harboring parasites with mutations in *pfK13/pfpm2/pfEXO* exhibited a 4.95-fold increased risk of treatment failure compared to those without these mutations (95%CI:1.06–28.75, $p<0.05$). After adjusting for general characteristics, including age, study site, parasite density, and BMI, the OR for treatment failure in patients with parasites carrying all three mutations (*pfK13, pfpm2, pfEXO*) increased to 7.71, with a statistically significant difference ($p<0.05$). These findings demonstrate a strong correlation between these combined genetic mutations and treatment failure, emphasizing the need for focused molecular monitoring and tailored therapeutic strategies.

The results of model selection using the Bayesian Model Averaging (BMA) method identified seven models based on three mutations in candidate genes to determine their roles and associations with treatment failure in parasites under combination therapy.

Table 3.7. Evaluation of Genetic Marker-Based Models for Predicting Uncomplicated Malaria Treatment Outcomes

Factors	Model 1	Model 2	Model 3	model 4	Model 5
Intercept	**0,00**	**-0,16**	**0,48**	**-0,27**	**-0,20**
pfK13 (C580Y)	0,58	0,61	-	0,55	0,57
pfpm2 (>1 bản sao)	-	0,27	-	-	0,27
pfEXO(E415G)	-	-	-	0,07	0,09
Number of variables	**1**	**2**	0	**2**	**2**
R^2	**0,18**	**0,253**	**0**	**0,183**	**0,258**
BIC	**-2,7**	**-2,17**	**0,000**	**0,60**	**1,06**
Posterior probability	**0,392**	**0,297**	**0,100**	**0,074**	**0,059**

The Bayesian models (Table 3.7) highlight the significant role of genetic mutations in treatment failure. The *pfK13 (C580Y*) mutation consistently appears as the most influential factor, included in the majority of models with high posterior probabilities. In contrast, the *pfpm2* copy number variation and *pfEXO (E415G)* mutation show less consistent associations, appearing in fewer models with lower impact on treatment outcomes.

Chapter 4. Discussion

4.1. Genetic variations and the mutation frequencies of key drug resistance genes in *P. falciparum* malaria parasites.

4.1.1. Prevelance of pfK13 mutation

The C580Y mutation is a prominent mutation in the *pfK13* gene, and its presence in our study aligns with findings from other countries in the Greater Mekong Subregion. The frequency of C580Y observed in this study (91.3%) is consistent with previous reports from Vietnam, indicating that this mutation is widespread and plays a central role in artemisinin resistance. Furthermore, the increasing frequency of C580Y over time, as reported in multiple studies, highlights the progressive selection pressure imposed by DHA-PPQ in Vietnam.

When compared to Africa, the prevalence of the C580Y mutation in Vietnam and the Mekong region is substantially higher. In Africa, the mutation has been reported at much lower frequencies, as artemisinin resistance is still emerging on the continent. This contrast underscores regional differences in drug resistance dynamics, driven by varying treatment policies, drug pressure, and parasite genetics.

The persistence and increasing prevalence of C580Y in Vietnam and neighboring countries emphasize the critical need for enhanced monitoring and the development of alternative treatment strategies to preserve the efficacy of ACT in the region. These findings are a call to action for sustained surveillance and collaborative efforts to combat artemisinin resistance globally.

4.1.2. Prevalance of pfpm2 copy number variation

The presence of multiple copies of the *pfpm2* gene, a key molecular marker for PPQ resistance, is a critical finding in this study, with 51.0% of samples exhibiting gene amplification. This is

particularly concerning as DHA-PPQ remains the primary first-line therapy for *P. falciparum* malaria in Vietnam. The high frequency of multiple pfpm2 copies, especially in Gia Lai (55.6%), reflects the increasing drug pressure exerted by widespread use of DHA-PPQ. Previous studies have established a strong association between *pfpm2* amplifications and PPQ resistance, which has been linked to treatment failures in Southeast Asia.

The prevalence of *pfpm2* amplifications in Vietnam, coupled with the widespread C580Y mutation in *pfK13,* suggests a dual resistance mechanism to both components of the DHA-PPQ regimen. This dual resistance threatens the continued efficacy of DHA-PPQ, highlighting the need for immediate action. Enhanced surveillance to monitor the spread and impact of *pfpm2* amplifications is essential, along with the exploration of alternative drug combinations or next-generation artemisinin-based therapies. Regional collaboration is also crucial to address the shared challenge of PPQ resistance in the Greater Mekong Subregion. Without timely intervention, the increasing prevalence of *pfpm2* amplifications could undermine the effectiveness of DHA-PPQ as a first-line treatment, necessitating urgent adjustments in malaria treatment policies.

4.1.3. Prevalence of E415G mutation

The E415G mutation in the *pfEXO* gene, a molecular marker associated with PPQ resistance, was detected in 76.1% of samples in this study. This high prevalence aligns with findings from other regions of the Greater Mekong Subregion, where PPQ resistance has been increasingly documented. The mutation frequency varied across study sites, with Gia Lai exhibiting the lowest frequency (70.0%) and Đắk Lắk the highest (85.9%). The significant variation (p=0.014) suggests regional differences in drug pressure and resistance dynamics.

E415G has been implicated in the mechanism of PPQ resistance when combined with *pfpm2* amplifications. The high prevalence of this mutation in Vietnam, alongside the widespread use of DHA-PPQ as the first-line treatment, raises concerns about the durability of the regimen. The co-occurrence of E415G and multiple *pfpm2* copies in many samples suggests a synergistic resistance mechanism, which could lead to increased treatment failures.

The findings emphasize the need for proactive measures, including enhanced surveillance of resistance markers like E415G, to assess the efficacy of DHA-PPQ. Additionally, research into alternative drug regimens and next-generation combination therapies should be prioritized to ensure effective malaria treatment in Vietnam. Without timely intervention, the high prevalence of E415G and its association with PPQ resistance could compromise the long-term success of current malaria control efforts.

4.1.4. Prevalence of combine genotype

A significant proportion of *P. falciparum* parasites in the study sites carry both C580Y, associated with artemisinin resistance, and pfpm2/E415G, linked to piperaquine resistance. This co-occurrence strongly suggests a multidrug-resistant profile and aligns with reports from other malaria-endemic regions where KEL/PLA lineages (commonly designated by the presence of *pfK13*—including C580Y—and *pfpm 2–3* amplifications) have proliferated. Such lineages have been widely documented in parts of the Greater Mekong Subregion (notably in Cambodia and Thailand), contributing to declining efficacy of DHA–PPQ and prompting changes in national treatment policies.

Given that DHA–PPQ remains the primary therapy in many endemic settings, the spread of these multidrug-resistant strains poses a serious threat to malaria control and elimination efforts. Other countries facing similar KEL/PLA expansions have observed rising rates of treatment failure, underscoring the importance of both TES and molecular surveillance to detect, track, and respond to resistance patterns in real time. Strengthening drug quality assurance and patient adherence to prescribed regimens, alongside robust vector control measures, remains critical to slow the expansion of multidrug resistance. Failure to respond promptly may lead to further compromise of frontline treatments and reverse many of the recent gains in reducing malaria morbidity and mortality.

4.2. DHA–PPQ Treatment Outcomes in Patients with Uncomplicated *P. falciparum Malaria*

In this study, the ACPR, which indicates successful treatment with DHA-PPQ, observed in this study was 50.8%, which is significantly lower than the rates reported in earlier studies in Vietnam and neighboring countries. Previous studies conducted in Vietnam typically reported ACPR rates above 95% during earlier periods of DHA-PPQ use, indicating better treatment efficacy before the widespread emergence of drug resistance.

The sharp decline observed in this study reflects the growing prevalence of key resistance markers, such as the C580Y mutation in *pfK13* and amplifications in *pfpm2* and *pfEXO,* which have been increasingly documented in recent years. Comparatively, studies in Cambodia and Laos, where similar resistance dynamics are prevalent, have also reported declining ACPR rates, in some cases dropping below 60%, similar to our findings.

Globally, regions without significant piperaquine resistance, such as sub-Saharan Africa, have reported much higher ACPR rates exceeding 90%, highlighting the stark contrast between resistance levels in Southeast Asia and other parts of the world. This comparison underscores the urgent need for Vietnam to adapt its treatment policies to address the declining efficacy of DHA-PPQ and to prevent further spread of resistance. Enhanced surveillance and alternative therapeutic strategies are critical to mitigating this challenge.

4.3. Factors Influencing the Efficacy of DHA–PPQ in the Treatment of Uncomplicated *P. falciparum* Malaria

Our findings reveal that anthropometric factors (e.g., age, BMI, study location) did not significantly affect the therapeutic efficacy of dihydroartemisinin–piperaquine (DHA–PPQ); however, resistance-associated genetic mutations in *pfK13, pfpm2, and pfEXO* emerged as the principal factors driving treatment failure. Notably, patients harboring these mutations demonstrated a 4.95-fold higher risk of failing therapy compared to those without such mutations, and once data were adjusted for potential confounders—such as parasite density and BMI—this risk rose to an odds ratio (OR) of 7.71.

Compounding this issue, the overall treatment success rate (as measured by adequate clinical and parasitological response, ACPR) was found to be only 50.8%, signaling a worrisome decline in the real-world performance of DHA–PPQ. This reduced efficacy underscores the practical clinical impact of multidrug resistance (MDR). Mechanistically, *pfK13* mutations undermine artemisinin's rapid parasite clearance, while variations in *pfpm2* and *pfEXO* diminish the potency of piperaquine, thereby compromising both key

components of this combination therapy. When multiple resistance mutations co-occur, they can have an additive—or even synergistic—effect, leading to pronounced reductions in cure rates.

From a public health standpoint, these results underscore the urgency of monitoring both clinical outcomes and resistance markers in a coordinated manner. Routine TES, along with molecular surveillance, enable early detection of declining efficacy. This, in turn, informs the timely revision of treatment guidelines, potentially including alternative combination therapies or additional interventions. Meanwhile, ensuring high drug quality, bolstering patient adherence, and maintaining comprehensive vector control measures remain crucial elements in mitigating the spread of multidrug-resistant *P. falciparum*. If resistance continues to intensify unchecked, the dwindling effectiveness of DHA–PPQ could significantly undermine regional malaria control and the broader goal of malaria elimination.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

1- Genetic variations in several drug-resistance genes of malaria parasites have been identified, with a significant increase in the mutation frequency of genes conferring resistance to dihydroartemisinin-piperaquine surveyed provinces. The prevalence and distribution of single and complex drug-resistance mutations vary across malaria-endemic provinces.

2- The therapeutic efficacy of DHA-PPQ in treating uncomplicated malaria caused by *Plasmodium falciparum* has significantly declined in certain malaria-endemic provinces in Vietnam, specifically as follows:

a. DHA-PPQ has shown reduced effectiveness in clearing malaria parasites and preventing recurrence, with a day-3 positivity rate of 21.7% and a cumulative recurrence rate of 22.6%.

b. The efficacy outcomes were low, particularly in Bình Phước and Đắk Nông, at 47.7% and 57.9%, respectively.

3- Several factors influencing the efficacy of dihydroartemisinin-piperaquine in treating uncomplicated P. falciparum malaria have been analyzed. Genetic mutations associated with drug resistance, particularly C580Y, E415G, and increased copy numbers of the *pfpm2* gene, have been linked to treatment failure, with patients harboring these mutations being at a higher risk of therapeutic failure compared to those without such mutations.

RECOMMENDATION

1- Enhance the surveillance of genetic mutations in malaria parasites, focusing on mutations *in pfpm2, pfEXO, and pfK13* genes. Large-scale genetic monitoring programs should be implemented to assess the distribution and frequency of these mutations.

2- Strengthen national malaria control strategies by integrating molecular data into decision-making processes to improve treatment efficacy and address emerging drug resistance.

3- Promote research initiatives aimed at understanding the mechanisms of drug resistance and identifying alternative therapeutic regimens to counteract resistance in malaria-endemic regions.

LIST OF PUBLICATIONS RELATED TO THE DISSERTATION

1- **Thu Huyen Thi Tran**, Bui Thi Thu Hien, Nguyen Thi Lan Dung, Nguyen Thi Huong, Tran Thanh Binh, Nguyen Van Long, Nguyen Dang Ton, 2024, Evaluation of DHA-PPQ Efficacy and Molecular Marker in Uncomplicated Falciparum Patients: A Study across Binh Phuoc and Dak Nong, Vietnam*, Medicina*, 60(6), 1013.

2- **Tran Thi Thu Huyen**, Le Van Khanh, Bui Thi Thu Hien, Nguyen Thi Lan Dung, Nguyen Van Long, Nguyen Dang Ton, 2023, Reporting the impact of artemisinin resistance: Molecular surveillance of pfK13 and pfEXO mutations in *Plasmodium falciparum* in Southern provinces of Vietnam, *Tạp chí Công nghệ sinh học Việt Nam* 21(3), 393–405.