MINISTRY OF EDUCATION AND TRAINING **MINISTRY OF HEALTH**

National Institute of Malariology, Parasitology, and Entomology

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RESEARCH ON SOME MOLECULAR MARKERS EPEDIMIOLOGY CHARACTERISTICS OF K13 GENES MUTATION AND RESPONSE OF *Plasmodium falciparum* TO DIHYDROARTEMISININ-PIPERAQUIN PHOSPHATE REGIMEN IN SOME MALARIA ENDEMIC AREAS IN VIETNAM

Major: Epidemiology

Code: 972.01.17

SUMMARY OF MEDICAL Ph.D THESIS

Ha Noi, 2022

THE STUDY HAS BEEN COMPLETED IN NATIONAL INSTITUTE OF MALARIOLOGY - PARASITOLOGY AND ENTOMOLOGY

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Counter-argument1: .....

Name of work unit.....

Counter-argument 2: .....

Name of work unit.....

Counter-argument 3: .....

Name of work unit.....

The thesis will be defended before the Academy-level doctoral thesis quality examination Council in National Institute of Malariology - Parasitology and Entomology at …. a.m. on December …, 2022.

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1. National Library of Vietnam
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LIST OF THESIS-RELATED PUBLICATIONS OF THE AUTHOR

1. Do Manh Ha, Truong Van Hanh, Bui Quang Phuc *et al.* Frequency of mutations in the K13 gene related to artemisinin resistance of *P. falciparum* populations in some malaria-endemic areas in Vietnam in 2016-2018. *Journal of Malaria and Parasitic Diseases Prevention,* No. 3 (129)/2022:36-43.
2. Do Manh Ha, Truong Van Hanh, Bui Quang Phuc *et al.* Therapeutic efficacy of dihydroartemisinin - piperaquine phosphate in treatment for uncomplicated *Plasmodium falciparum* patients in the period 2016-2018. *Journal of Malaria and Parasitic Diseases Prevention*. No. 3 (129)/2022:21-29.
3. Do Manh Ha, Truong Van Hanh, Bui Quang Phuc, Huynh Quoc Phong *et al.* Therapeutic efficacy of dihydro-artemisinin-piperaquine phosphate on uncomplicated *Plasmodium falciparum* patients in Binh Phuoc 2017. *Journal of Malaria and Parasitic Diseases Prevention.* No. 3 (129)/2022:63-69.

INTRODUCTION

Artemisinin and its derivatives are important components in the Artemisinine-based Combination Therapies (ACTs) medicines for the treatment of drug-resistant *Plasmodium falciparum* (*P. falciparum*) malaria. The World Health Organization (WHO) has recommended the use of ACTs in many countries, including Vietnam. In Vietnam, the of dihydroartemisinin-piperaquine (DHA-PPQ) combination was selected for inclusion in the treatment of malaria caused by *P. falciparum*, during period from 2005 to 2010. The adequate clinical and parasitological response rate (ACPR) from 97.8% to 100%, but then parasites on positive D3 increased rapidly from in 2012-2015 of 30.6%; 36% (Binh Phuoc), 24.1% (Gia Lai), 17.4% (Khanh Hoa), respectively.

Recently, some mutant in the K13 gene have been identified as molecular markers closely related to *P. falciparum* artemisinin resistance.

With the conclusion to assess the situation of drug-resistance by *in vivo*, *in vitro*, and artemisinine resistance-related K13 molecular markers in *P. falciparum* population, the thesis topic "**Research on some molecular marker epidemiology characteristics of K13 genes and response of *Plasmodium falciparum* to dihydroartemisinin-piperaquine phosphate regimen in some malaria endemic areas in Vietnam**" was carried out with the following objectives:

Objectives of the study:

1. Description of some molecular markers epidemiological characteristics in K13 gene mutation of *Plasmodium falciparum* in Binh Phuoc, Gia Lai, Khanh Hoa, Ninh Thuan, Quang Tri provinces from 2016 to 2018;
2. Evaluation of the response of the *Plasmodium falciparum* parasite to the dihydroartemisinin - piperaquin phosphate at the study sites.
3. Evaluation of the susceptibility of *Plasmodium falciparum* to antimalarial drugs by *in vitro* testing in Gia Lai.

THESIS' SCIENTIFIC, NOVEL AND PRACTICAL POINTS

- In this study, we detected of eight K_{13} gene mutants, including two confirmed validated mutations for artemisinin resistance (C580Y and P553L), one related mutant (C496F), and 5 unidentified mutants (H384Q, G638E, G639D, Y511H, K503N) with a low rate, there is the first point mutant in Vietnam. This study found 3 provinces with resistance ART identified: Binh Phuoc, Gia Lai, Khanh Hoa (with positive D3 > 10% and K13 at the sites of ART resistance > 5%) and one province need to change drug policy-Binh Phuoc (as ACPR less than 90%). Currently, all these 3 provinces have been replaced of DHA-PPQ with Pyramax.

- Determining the distribution of K13 mutant genotypes in malaria hyperendemic provinces is a new aspect of the plan to contain the spread of artemisinin-resistant *P. falciparum* populations and is seen as a major problem, and a global health priority, because artemisinin resistance is a major threat to the global malaria control as well as in Vietnam;

- This is one of rare studies on the distribution of K13 gene mutations in many malaria endemic areas combined with *in vivo* drug efficacy monitoring and *in vitro* drug sensitivity testing. This study showed that 3 provinces with ART resistance, included of: Binh Phuoc, Gia Lai, Khanh Hoa (with D3 > 10% and K13 mutants at the sites of ART resistance > 5%) and 1 province need to change drug Which is Binh Phuoc province (ACPR less than 90%).

STRUCTURE OF THE THESIS

- The thesis covers 135 pages, including: Introdution with 2 pages, General overview with 32 pages; Research subjects and methods with 28 pages; Results with 36 pages; Discussion with 34 pages; Conclusion with 2 pages; Recommendations with 1 page. The thesis has 23 figures, 42 tables, and total of 114 references, in which 56 publications have been published in recent 5 years.

Chapter 1
OVERVIEW

1.1. Malaria in the world and Vietnam

According to the World Health Organization report, In 2019 there were an estimated 229 million malaria cases in 87 malaria-endemic countries, a decrease of 3.78% compared to 2000 (229/238 million). The prevalence of malaria per 1,000 population at risk decreased from 80 (in 2000) to 58 (in 2015) and 57 (in 2019). Between 2000 and 2015, the global incidence of malaria fell by 27.00%, and from 2015 to 2019 the number of cases decreased by less than 2.00%, indicating a slower rate of decline after 2015 [107]], [108].

In Vietnam, according to malaria control program's data in 2015 .There are 9331 patients in the whole country, mainly in the Central Coast, Central Highlands, and Southeast [28].

1.2. Glossary of terms related to drug resistance

To date, WHO has officially recognized drug resistance for 3 out of 5 types of malaria parasites that cause disease in humans. These are *P. falciparum*, *P. vivax, P. malariae*, of which the most significant is multi-drug resistant *P. falciparum* and the only species with reduced sensitivity and resistance to artemisinin and derivatives. However, in clinical practice, there is still confusion between the terms "drug resistance" and "treatment failure" [106]. In 2018, the definition of partial resistance to artemisinin (artemisinin partial resistance) was given by WHO when there was a delay in parasite clearance, due to partial resistance to artemisinin on the ring body.

1.3. K13 gene mutation

- The K13 gene is considered a genetic marker related to the *P.falciparum* malaria parasite resistant to artemisinin and its derivatives. Structurally, the K13 gene is an exon encoding the Kelch 13 protein with a length of 726 amino acids
- Up to now, WHO Report (2018) [105] has confirmed: 9 K13 gene mutation sites have value for determining artemisinin resistance and 11 mutation sites have identified value related to artemisinin resistance (Table 1.1)

Table 1.1. K13 gene mutations are associated with artemisinin resistance.

Identified resistance K13 markers		**Indicators K13 candidates/related**	
F446I	P553L	P441L	G538V
N458Y	R561H	G449A	V568G
M476I	C580Y	C469F	P574L
Y493H		A481V	F673I
R539T		P527H	A675V
I543T		N537I	

1.4. Status of *Plasmodium falciparum* parasite resistance to antimalarial drugs

1.4.1. In the world

The situation of drug-resistant parasites is very complicated, with 73/95 countries and territories reporting drug-resistant P. falciparum. The frequency and level of drug-resistant P. falciparum were highest in areas with complicated epidemiology such as Thailand, Cambodia, and Vietnam [59]. Some Central American countries, Haiti [93].

1.4.2. In Viet Nam

In 2009, the first case of early failure of *P. falciparum* with Arterakin appeared in Dak Nhau commune, Bu Dang district, Binh Phuoc province and then in Phu Thien district, Gia Lai in 2010 [29]. Regular monitoring of the therapeutic efficacy of DHA-PPQ has discovered more points with a D3 ≥ 10% asexuality rate, such as Gia Lai (2010), Dak Nong and Quang Nam (2012) [4], [15]].

1.5. Therapeutic efficacy, tolerability and safety of the DHA-PPQ . combination

Many studies by Ta Thi Tinh et al. (2011) [29], Bui Quang Phuc et al. (2013-2015) [15], [16] and Tran Tinh Hien et al (2014) [60], [61) at Bu Dang (Binh Phuoc) and Huynh Hong Quang et al (2014-2017) [21], [22], [23] in Tuy Duc (Dak Nong), Phu Thien (Gia Lai), Nam Tra My (Quang Nam) with ACPR rate from 91.2-100%, but survival rate of clonal parasites on D3 ranged from 14.7 to 44%.

Chapter 2
RESEARCHING METHODS

2.1. Description of some some molecular pathological genology and mechanism characteristics K13 gene of *Plasmodium falciparum* in Binh Phuoc, Gia Lai, Khanh Hoa, Ninh Thuan, Quang Tri province with severe malaria among period from 2016 to 2018

2.1.1. Subjects, time, and place of the study

2.1.1.1. Research subjects

- Absorbent blood samples were collected from uncomplicated *P.falciparum* malaria patients

Criteria for selecting disease:

+ Simple infection with *P. falciparum*; Regardless of age, gender, ethnicity, voluntarily participated in the study.

Exclusion criteria

+ Parasite *P. falciparum* infection with other species

2.1.1.2. Research time

Research period: From 2016 to 2018

2.1.1.3. Research location:

The study conducted in 5 provinces including: Binh Phuoc, Gia Lai, Khanh Hoa, Ninh Thuan and Quang Tri province collecting medical records

Research and analysis of samples in the laboratory: At the laboratory of molecular biology, Department of Molecular Biology, Institute of Malaria - Parasites - Central Government.

2.1.2. Research Methods

2.1.2.1. Research method design

Descriptive study with analysis of the K13 gene mutation of *P.falciparum* in the laboratory.

2.1.2.2.Sample size and sampling method

Sample size: Calculated according to the following formula:

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

Substituting the values into the above formula, a sample size of n = 288 samples in 5 provinces is calculated.

Sampling method: The sample was collected non-randomly, from *P. falciparum* malaria patients alone.

2.1.2.3. research content

- Sample collection: Collect blood samples of patients infected with P. falciparum parasite on Whatman 3MM blotting paper.
- Perform K13 gene sequencing analysis by Sanger method.
- Analyze the results to determine the location of mutation points.

2.1.2.4. Techniques used in the study:

- Collect blood samples from malaria patients infected with *P. falciprum* on Whatman 3MM blotting paper [5].
- Separate DNA with kit.
- PCR reaction cloning K13 gene with primer sequence designed by Arey et al. 2014 and sequencing K13 gene by Sanger method [40].

2.1.2.5. Rating metrics:

- Rate, frequency, location of gene mutations, by age group, by gender at the study sites.
- Compare the rate of mutation, mutation type between study sites

2.1.2.6. Methods of data analysis and processing:

- Read and analyze K13 gene sequence using Bioedit V.7.0.5.3 bioinformatics software.

2.2. Evaluation of the response of the malaria parasite *Plasmodium falciparum* to the drug dihydroartemisinin - piperaquin phosphate at the study sites

2.2.1. Subjects, time and place of the study

2.2.1.1. Research subjects

- Patients diagnosed with uncomplicated *P. falciparum* malaria who met the inclusion criteria were included in the in vivo trial study subjects [101].
- Research drug: Dihydroartemisinin - piperaquine.

2.2.1.2. Research location

Conducted in 5 provinces including: Binh Phuoc, Gia Lai, Khanh Hoa, Ninh Thuan and Quang Tri

2.2.1.3. Research time

Research carried out from 2016 to 2018

2.2.2. Research Methods

2.2.2.1 Study designing

Non-randomized, non-controlled clinical trial.

2.2.2.2. Sample size and sampling method

- Sample size for the study: The sample size was calculated based on the WHO sample size determination table, 2009 [101].

The overall sample muscle was 157 patients.

2.2.2.3. research content

- Screening eligible subjects for inclusion in the study
- Clinical symptom study
- Paraclinical research

2.2.2.5. Techniques used in the study

- Techniques for taking blood smears, thick and thin blood smears [6].
- Technique of counting parasite density:
- Technical process and patient monitoring
- Molecular biology techniques to distinguish relapse and reinfection 2.2.2.6. Indicators and variables in the study
- Time to clean parasites;
- Time out / fever cut off;
- Classification of treatment outcomes according to WHO (2009).

2.2.2.6. Data entry and analysis

- The collected research data was synthesized, analyzed and processed according to in vivo software version 7.1 Pascal Ringwald, WHO (2009) [101].

2.3. Evaluation of the susceptibility of *Plasmodium falciparum* to antimalarial drugs by in vitro technique in Gia Lai

2.3.1. Researching objects, places and time.

2.3.1.1. Research subjects

- Patients diagnosed with uncomplicated *P. falciparum* malaria who met the criteria were selected in the in vitro study [102].
- Research drug: Artesunate powder; dihydroartemisinin; piperaquine phosphate; chloroquine phosphate.

2.3.1.2. Research location

Research in KrongPa district, Gia Lai province.

2.3.1.3. Research time

Research carried out from 2016 to 2018

2.3.2. Research Methods

2.3.2.1 Study design

In vitro analytical study to evaluate drug efficacy in the field laboratory.

2.3.2.2. Sample size and sampling method

- Minimum sample size is 30 patients.

2.3.2.3. research content

Culturing *P. falciparum* parasites according to 48h drug testing procedures in the field

2.3.2.4. Techniques used in the study

Determination of susceptibility of malaria parasites to antimalarial drugs (in vitro) [57],[92],[98]

2.3.2.5. The index variables in the study

- Determine the concentration of drugs that inhibit 50%, the development of malaria parasites.

2.3.2.6. Data entry and analysis

- Determination of 50% inhibitory concentration (IC50), parasite growth will be calculated using analysis and reporting method (IVART) software developed online by WWARN.

2.4. Methods to control noise and limit errors

- Before conducting the research, the principal investigator must repeat and re-train the sequence of research steps;
- Select research subjects strictly according to research criteria;
- During data analysis and report writing: Closely monitor data collection in the field, clean data on completed questionnaires, encrypt and remove unreliable data .

2.5. Ethics in research

The topic was approved by the Research Ethics Board and approved the outline of the Central Institute of Malaria-parasites-CT;

Chapter 3
RESEARCHING RESULTS

3.1. Description of some molecular pathological mechanism characteristics K13 gene mutation of *Plasmodium falciparum in* 5 provinces of Binh Phuoc, Gia Lai, Khanh Hoa, Ninh Thuan, Quang Tri with severe malaria in 2016 – 2018

3.1.1. General characteristics of patients infected with *P.falciparum* included in K13 gene mutation analysis

A total of 292 isolates from *P. falciparum* malaria patients were collected from 5 provinces

Table 3.1. General characteristics of the group of patients participating in the study

Characteristics	Binh Phuoc n=39	Gia Lai n=108	Khanh Hoa n=52	Ninh Thuan N= 44	Quang Tri N=49
Gender	**Amount**	**Amount**	**Amount**	**Amount**	**Amount**
male	*26*	*97*	*35*	41	*31*
Female	*13*	*11*	*17*	3	*18)*
Age group	**Amount**	**Amount**	**Amount**	**Amount**	**Amount**
< 15 years old	*5*	*12*	*21*	*11*	*11*
≥ 15 years old	*34*	*96*	*31*	*33*	*38*
Average age	*26,8 ± 12,4*	*26,9± 9,9*	*25,9 ± 18,2*	*26,8 ± 14,3*	*27,1 ± 16,8*

Comment:

Analyzing the characteristics of the study group of patients, we found that male patients accounted for the majority. The majority of patients participating in the study were 15 years old or older, the highest was Gia Lai (88.89%) and the lowest was Khanh Hoa (59.6%).

3.1.2. Characterization of mutation sites on the K13 gene of P. falciparum on analyzed samples

The analysis results identified 8 types of K13 gene mutations including: C580Y mutation detected in all 5 provinces, P553L gene mutation detected in Gia Lai and Khanh Hoa provinces, C469F mutation detected in Gia Lai and Khanh Hoa provinces. In Gia Lai, other gene mutations include H384Q, K503N, Y511H, G638E, G639D, these are the mutation sites whose role has not yet been determined.

3.1.3. The rate of K13 gene mutation of the 5 provinces in which the study was conducted

Table 3.17. Results of K13 gene mutation rate at the study site

TT	Province	Analytical samples	Samples with K13 gene mutation		P
			Amount	%	
1	Binh Phuoc	39	38	97,44	0,00063
2	Gia Lai	108	67	62,04	
3	Khanh Hoa	52	17	32,69	
4	Ninh Thuan	44	3	6,82	
5	Quang tri	49	10	20,41	
	Total	**292**	**135**	**46,23**	

Comment:

The combined results obtained the overall K13 gene mutation rate of 135/292 (46.23%). The rate of P.falciparum K13 gene mutation among the studied provinces has a statistically significant difference p<0.05 Table 3.16, Figure 3.7

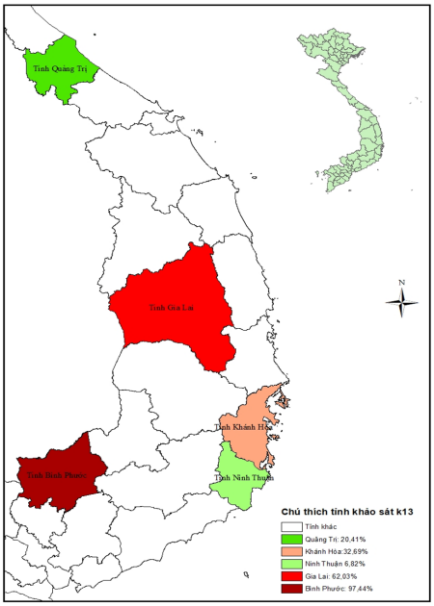

Figure 3.7. Map of the distribution of K13 gene mutations in *P.falciparum* parasites at study sites

Table 3.18. K13 gene mutation characteristics of 5 studied provinces

Genotypes	Amount	Rate %	Classification
Mutation P553L	18	6,16	Determine resistance
Mutation C580Y	103	*35,27*	Determine resistance
Mutation C469F	4	1,37	Related/Candidate
MutationH384Q	1	0,34	Role not determined yet
Mutation Y511H	5	1,71	Role not determined yet
Mutation K503N	2	0,68	Role not determined yet
Mutation G638E	1	0,34	Role not determined yet
Mutation G639D	1	0,34	Role not determined yet
Wild type	157	53,77	Sensitive to ART
Total	**292**	**100%**	

Comment:

The C580Y mutation type accounted for the highest rate of 35.27% (103/292), followed by the P553L point accounted for 6.16% (18/292) these are also 2 points identified as ART resistance, the C496F mutation point. detected only with a small percentage (1.37%). In addition, there are 5 other mutant sites whose roles have not been determined.

3.1.5. Comparison of K13 gene mutation rates of *P.falciparum* populations at study sites.

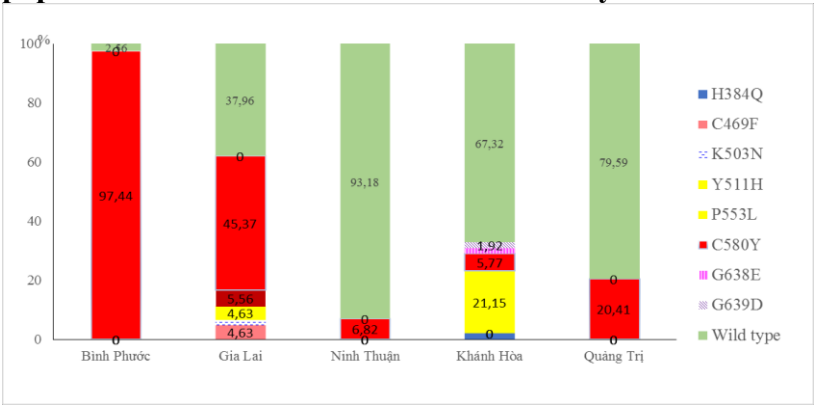

Figure 3.8. The chart comparing the rate of K13 mutations detected at the study sites

The mutation C580Y was detected in all 5 provinces, the highest in Binh Phuoc with the frequency of mutant genotypes accounting for 97.44% (38/39 samples) and the low rate in Khanh Hoa 5.77%

(3/3). 52 samples). Artemisinin resistance mutation P553L was detected in 2 provinces, Gia Lai 5.56% (6/108 samples) and in Khanh Hoa 21.15% (11/52 samples). The C496F mutation was detected only in Gia Lai province with the rate of 4.63% (5/108 samples). Some other unspecified resistance mutations were detected such as K503N Y511H, H384Q, G638E, G639D 1.92% with low rate.

3.2. Evaluation of the response of the malaria parasite *Plasmodium falciparum* to the drug dihydroartemisinin - piperaquin phosphate at the study sites

Out of a total of 292 *P. falciparum* malaria patients, 201 eligible patients were selected to be included in the *in vivo* study.

3.2.1. General characteristics of the population of the study group

Table 3.21. General characteristics of the group of patients participating in the study

Characteristics	Binh Phuoc n= 39	Gia Lai n=48	Khanh Hoa n= 43	Ninh Thuan n=40	Quang Tri n=31
Gender	**Amount**	**Amount**	**Amount**	**Amount**	**Amount**
male	26	46	29	38	18
Female	13	2	14	2	13
Age group					
< 5	0	0	1	1	2
≥ 5 - < 15	5	1	17	9	6
≥ 15	34	47	25	30	23
Temperature (^{0}C)	38,7 ± 0,6	38,7 ± 0,9	38,8 ± 0,9	38,9 ± 0,4	38,8 ± 0,8
Weight (kg)	51 ± 10,7	53,4 ± 8,2	37,6 ± 13,4	42,8 ± 13,8	40,4 ± 12,7
Mật độ KST	8.030/µL (1128-97764)	10.770/µL (1019-98765)	11.736/µL (1020-99555)	26.530/µL (3111-97666)	11.087/µL (1441 - 98234)

Comment:

Males are higher than females in the structure at all TES sites, most of the patients are adults (≥ 15 years old), All patients had fever or a history of fever within 24 hours. The average density of *P. falciparum* parasites in the provinces ranged from 8030 to 26530/µL

3.2.3. Time to clear parasites and cut fever after DHA-PPQ . treatment

Table 3.24. Efficacy in clearing *P. falciparum* and reducing fever

Analytic content	Binh Phuoc	Gia Lai	Khanh Hoa	Ninh Thuan	Quang Tri
Malaria parasite density/µl Day D_0	8030	10770	11736	26530	11087
Parasite clearance time (hours)	52,1 ± 20,1	45,5 ± 22,2	38,1 ± 16,6	31,2 ± 11,1	24,6 ± 1,5
Day temperature D0 (^{0}C)	38,7 ± 0,6	38,7 ± 0,9	38,8 ± 0,9	38,9 ± 0,4	38,8 ± 0,8
Fever clearance time (hours)	30,2 ± 12,7	33,3 ± 14,9	31,4 ± 11,5	30 ± 11,5	34,2 ± 12,8

Comment:

After treatment with DHA-PPQ, the mean time of parasite clearance was 52.1 ± 20.1 hours, respectively; 45.5 ± 22.2 hours; 38.1 ± 16.6 hours; 31.2 ± 11.1 hours; 24.6 ± 1.5 hours. At the same time, the time to stop fever was 30.2 ± 12.7 hours, 33.3 ± 14.9 hours, 31.4 ± 11.5 hours and 34.2 ± 12.8 hours in Binh Phuoc, Gia Lai, respectively. Lai, Khanh Hoa, Ninh Thuan and Quang Tri.

Table 3.25. Survival rate of asexual parasites after 72 hours (D3)

Parasite clearance time	Binh Phuoc	Gia Lai	Khanh Hoa	Ninh Thuan	Quang Tri
Malaria parasite density/µl Day D_0	8030	10770	11736	26530	11087
The cases can be days D_1	35/39 (89,7%)	38/48 (79,2%)	37/43 (86,1%)	17/40 (42,5%)	6/31 (19,4%)
The cases can be days D_2	27/39 (69,2%)	24/48 (50,0%)	25/43 (58,1%)	1/40 (2,5%)	0/31 (0,0%)
The cases can be days D_3	16/39 (41,0%)	7/48 (14,6%)	8/43 (18,6%)	0/40 (0%)	0/31 (0%)

Comment:

Analysis of the survival rate of the clonal parasites after DHA-PPQ treatment at day D3 since the first dose of DHA-PPQ showed that at the monitoring points of Binh Phuoc, Gia Lai, Khanh Hoa provinces, the prevalence of parasites D3 day >10% is 41.0% (16/39), 14.6% (7/48), 18.6% (8/43) respectively. Ninh Thuan and Quang Tri have no cases of parasites. date D3.

Table 3.26. Compare parasites on day D3 with mutant K13 gene at study sites.

Province	**Samples with K13 gene mutation**		**Total**
	Parasite D3 (%)	**No parasites** D3 (%)	
Binh Phuoc n=39	16 41%	22 56,4%	38
Gia Lai n=48	7 14,6%	21 43,7%	28
Khanh Hoa n= 43	8 18,6%	8 18,6%	16
Ninh Thuan n=40	0 0%	2 5,0%	2
Quang Tri n=31	0 0%	10 32,6%	10
Total	31	63	**94**
	94		

Comment:

Among 201 patients followed up to day D3 of 5 provinces of Binh Phuoc, Gia Lai, Khanh Hoa, Ninh Thuan, Quang Tri, all 31 patients with parasites on day D3 all had K13 gene mutations determining ART resistance (24). .C580Y, 7.P553L) respectively in the provinces of Binh Phuoc 41%, Khanh Hoa 18.6% and Gia Lai 14.6% and 63/94 patients with K13 gene mutation but no parasite on D3. Thus, the provinces of Binh Phuoc, Gia Lai, Khanh Hoa have a rate of parasitemia on day D3 greater than 10%

associated with K13 mutation at sites where ART resistance is > 5% identified as ART resistant regions.

Table 3.27. Genotypic characteristics of patients with parasites on day D3

Genotypes	Binh Phuoc n=16	Gia Lai n =7	Khanh Hoa n =8	Total	Classification
Mutation P553L	0 (0 %)	1 (14,28%)	7 (87,5%)	8	Determine resistance
Mutation C580Y	16 (100%)	6 (85,72 %)	1 12,5%	23	Determine resistance
Mutation C496F	0 (0 %)	0 (0%)	0 (0 %)	0	Related/Candi date
Other Mutations	0 (0 %)	0 (0 %)	0 (0%)	0	Role not determined yet

Comment:

Among 31 patients with parasites on day D3 and with mutation score of K13 gene, there are 23 samples with C580Y mutation, 8 samples with P553L mutation. All of these mutations are classified as defining artemisinin resistance.

3.2.4. Efficacy of DHA-PPQ drug regimen for *P. falciparum* malaria

Table 3.28. Efficacy of DHA-PPQ drug regimen for *P.falciparum* malaria in studied provinces

Efficacy of DHA-PPQ	Binh Phuoc	Gia Lai	Khanh Hoa	Ninh Thuan	Quang Tri
	Amount *(%)*	Amount *(%)*	Amount *(%)*	Amount *(%)*	Amount *(%)*
ETF	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
LCF	2 (6,1%)	1 (2,2%)	0 (0%)	0 (0%)	0 (0%)
LPF	4 (12,2%)	0 (0%)	1 (2,6%)	0 (0%)	0 (0%)
ACPR	27 (81,8%)	44 (97,8%)	38 (97,4%)	33 (100%)	26 (100%)
Total	**33**	**45**	**39**	**33**	**26**

Comment:

Out of 176 patients were followed up for a full evaluation. The results show that the clinical response and adequate parasitemia (ACPR) in the two provinces of Ninh Thuan and Quang Tri are 100%, in Khanh Hoa and Gia Lai provinces, the effectiveness is still over 95%, 96.4% and 97 respectively. 4%, while in Binh Phuoc, this ACPR rate is only 81.8% (<90%) and the failure rate is over 10%, accompanied by late clinical failure (LCF) is 2 cases (6.1). %), late parasite failure (LPF) was 4 cases (12.2%). In particular, in all 5 provinces, there was no case of early treatment failure (ETF), in Khanh Hoa and Gia Lai there was only 1 case of late failure.

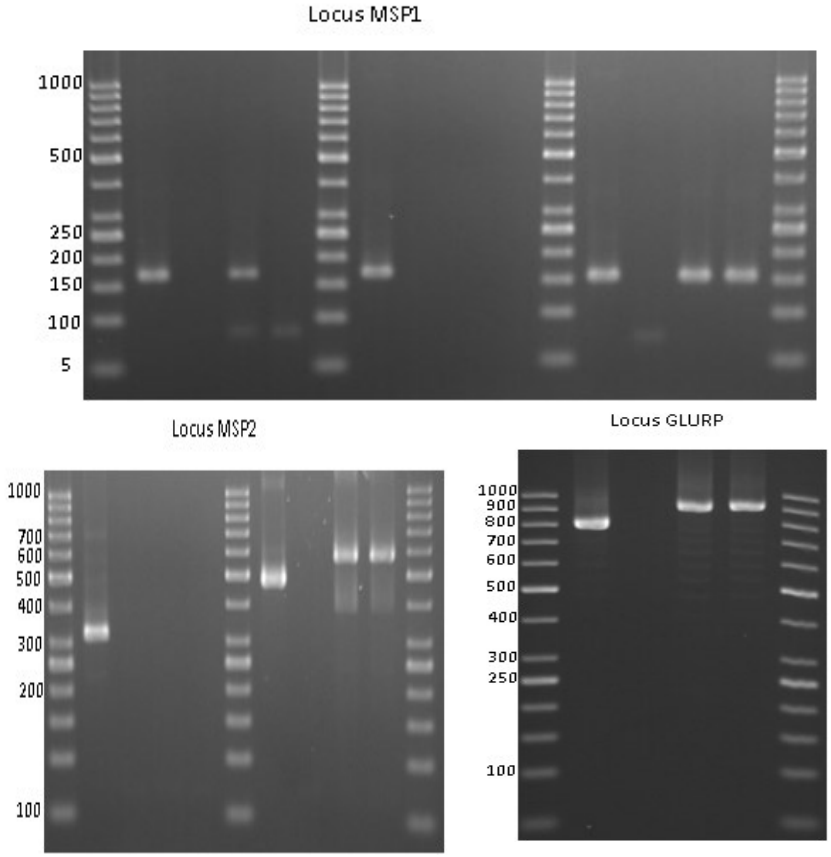

Figure 3.11. Electrophoresis images for analysis of relapse and reinfection

3.3. Evaluation of the susceptibility of the malaria parasite *Plasmodium falciparum* to antimalarial drugs by in vitro technique in Gia Lai

In 292 cases included in the analysis of K13 mutations, 54 isolates of *P. falciparum* were collected in Gia Lai to assess the susceptibility of the parasite to antimalarial drugs. Of which 54 samples were cultured, the rate of parasites developing into schizonts in control wells above 10% was 42/54 samples (77.7%).

3.3.1 Mutational characteristics of K13 gene of P. falciparum parasite on samples included in malaria drug testing

Table 3.31. Characterization of K13 gene mutation on successful culture samples

Genotypes	Amount n=42	Rate %	Classification
Mutation P553L	2	*4,76*	Determine resistance
Mutation C580Y	20	*47,62*	Determine resistance
Mutation C496F	3	*7,14*	Related/Candidate
Mutation Y511H	4	*9,52*	Role not determined yet
Wild type	13	*30,95*	Sensitive to ART
Total	**42**	***100***	

Comment:

- Through analysis, there were 29/42 samples detected many mutations: The mutation type C580Y accounted for 47.62% (20/42), P553L was 4.76% (2/42) were two mutations determining artemisinin resistance and candidate mutation C496F 7.14% (3/42). The K13 gene mutation at the Y511H position is 9.52% (4/42), the role is not yet determined

3.3.2. Evaluation of the susceptibility of the malaria parasite *Plasmodium falciparum* to some antimalarial drugs

The study results showed that the average IC50 of AS was 3.06 ± 3.10 nmol/L. In which, the lowest IC50 value is 0.41 nmol/L and the highest is 14.55 nmol/L; of DHA is 2.95 ± 2.19 nmol/L. The lowest IC50 value is 0.52 nmol/L and the highest is 9.94 nmol/L; of PPQ is 43.5 ± 24.9 nmol/L, the lowest IC50 value is 4.66 nmol/L and the highest is 105.8 nmol/L.

3.3.3. The relationship between K13 gene mutation of *P.falciparum* parasite and some antimalarial drugs in vitro in Gia Lai

Table 3.32. Comparison of K13 and IC50 gene mutations of artesunate on culture samples

Genotypes	Gen K13	IC_{50} (nmol/L)	P
Having K13 mutations associated with ART resistance	25/42	3,50	0,0034
Wild-type or K13 mutant is not associated with ART resistance	17/42	2,18	

Comment:

Through analysis, we found that 25/42 samples had K13 mutations related to ART resistance, these samples had IC50 index of 3.50 nmol/L and 17/42 samples did not have K13 mutations, these samples had only The average IC50 number is 2.18 nmol/L lower than the mutant samples. The difference was statistically significant with ($p < 0.05$)

Table 3.33. Comparison of K13 and IC50 mutations of dihydro artemisinin in culture samples

Genotypes	Gen K13	IC_{50} (nmol/L)	P
Having K13 mutations associated with ART resistance	25/42	3,53	0,0062
Wild-type or K13 mutant is not associated with ART resistance	17/42	2,37	

Comment:

Through analysis, 25/42 samples have K13 mutations related to ART resistance and derivatives, these samples have IC50 index of 3.53 nmol/L and 17/42 samples do not have K13 mutations in these samples. had an average IC50 of 2.37 nmol/L lower than the mutant samples. The difference was statistically significant with ($p < 0.05$).

Table 3.34. Comparison of K13 and IC50 gene mutations of piperaquin in culture samples

Genotypes	Gen K13	IC_{50} (nmol/L)
Having K13 mutations associated with ART resistance	25/42	41,81
Wild-type or K13 mutant is not associated with ART resistance	17/42	44,30

Comment:

Through analysis, we found that there were samples with K13 mutations related to resistance to ART and derivatives, these samples had an average IC50 index of piperaquine of 41.81 nmol/L and 17/42 samples did not have K13 mutations. or the K13 mutation was not associated with ART resistance, these samples had an average IC50 of 44.30 nmol/L higher than those with the mutation.

Chapter 4
DISCUSSION

4.1. Description of some epidemiological characteristics of K13 gene mutation of *Plasmodium falciparum* in 5 provinces of Binh Phuoc, Gia Lai, Khanh Hoa, Ninh Thuan, Quang Tri with severe malaria among period from 2016 - 2018

The study of the artemisinin-resistant K13 gene mutation on *P. falciparum* will help policymakers change drug policy. Data collected from 72 studies including K13 gene markers included 16,613 blood samples from 18 countries, samples from Myanmar 3842 (23.1%), Cambodia 3804 (22.9%), and Vietnam 2663 (16%). there was a gradual increase in the rate of K13 mutations, the lowest was 4.3% in 2005 (n=47) and the highest was 62.9% in 2018 (n=264) [54].

Comparison of the data of Nguyen Thi Minh Trinh et al. (2016) [32] and Nguyen Thi Lien Hanh et al. (2017) [9] with 55 simple infections of P. falciparum on K13 gene mutation for the rate 28/55 cases (50.9%) had mutation K¬13, in which mutation type C580Y dominated 21/28 cases (75%) and P553L was 8 cases (14.5%). Meanwhile, other mutations that have not yet been identified are P574L 2 cases (3.6%). Sequencing over 1,060 *P. falciparum* isolates from 3 malaria hotspots in Vietnam. The data showed that mutations in K13 genes including T474I, T493H, A539T, I543T, P553L, V568G, P574L and type C580Y with and without association with artemisinin resistance were also found. The frequency of K13 mutations ranged from 29% (222/767), 6% (11/188) and 43% (45/105) in the endemic areas of Binh Phuoc, Ninh Thuan and Gia Lai provinces, respectively. 32],[84]. The C580Y mutation has become the dominant genotype in recent years with the rate of 79.1% (34/43) isolated in Binh Phuoc in 2015 [75] and 63% (17/27) isolated in Gia Lai [27]. 75]. Simultaneously, the study of K13 gene mutation is also related to the sensitivity of the ring trophozoite of *P. falciparum* to dihydroartemisinin (DHA) in *in vitro* test and prolonged parasite clearance time in *in vivo* test.

During the period (2015-2016), mutation C580Y was the only type of K13 mutation detected in Gia Lai at 45/105 (43%). The association between K13 mutation and 50% clearance of the clinical parasite load was established and most patients infected with *P. falciparum* with mutations in the K13 allele had a 50% clearance time of 5 hours, in when this time in the group without K13 mutation but only wild type mutation, this difference was statistically significant ($p < 0.001$) [75].

Detecting genes related to drug resistance or current knowledge of the molecular cycle in the parasite cycle is more or less helpful in malaria control, especially the genes of Plasmodium spp. and of course it is not possible to guarantee that the genomic aspects that can lead to the cessation of malaria transmission play an important role in a more effective malaria control toolkit.

4.2. Evaluation of the response of the malaria parasite *Plasmodium falciparum* to the drug dihydroartemisinin - piperaquin phosphate at the study sites

Comparison with updated antimalarial efficacy data from WHO in vivo TES trials shows that in Cambodia, partial artemisinin resistance was first reported in 2008, but the retrospective analysis of molecular markers indicating partial resistance to artemisinin had emerged before 2001 and widespread resistance to ACTs [62], [94], [103].

Overall, despite the slow response to artemisinin in some GMS regions, ACTs are still effective in the treatment of P. falciparum malaria. Most patients with delayed parasite clearance are cured because the role of the concomitant medication is still effective. Routine monitoring needs to continue to ensure that currently recommended ACTs are effective and to promptly change drug policy when regulatory thresholds are reached. Evaluation of K13 gene mutations is an important aid in tracing partial artemisinin resistance as they are emerging. In the context of multidrug resistance in the GMS, eradication of P. falciparum is a matter of priority. The role of artemisinin resistance in the development or selection of partial resistance needs further evaluation in the future [107].

In Vietnam, the slow progress of parasite eradication after taking DHA-PPQ was first detected in Bu Dang district, Binh Phuoc province in 2009. Routine monitoring of the effectiveness of DHA-PPQ has also shown that other disease clusters are also present. similar in Phu Thien district, Gia Lai province (2010), Tuy Duc and Cu Jut districts, Dak Nong province (2011), Nam Tra My district, Quang Nam province (2012), Khanh Vinh district, Khanh Hoa province (2014)) and Ninh Son district, Ninh Thuan province (2015) [12],[25],[33].

TESs performed with DHA-PPQ from 2010-2014 also found a therapeutic effect of over 95%, although the percentage of D3-positive clones was up to 36%. However, a 2015 study in Binh Phuoc reported a high failure rate of over 10% after DHA-PPQ treatment and was confirmed by WHO to have the emergence of piperaquine resistance. In 2016, a high failure rate with DHA-PPQ was reported in Dak Nong province. More recently, studies have

shown more severe treatment failure and associated molecular markers of resistance to both artemisinin and the conjugate piperaquine in neighboring provinces from this resistant "oil slick". In 2016-2017, late treatment failure rates in Dak Nong and Binh Phuoc were 27% and 56%, respectively [86]. Research data from Krong Pa district, Gia Lai and Krong Nang district, Ea Kar, Dak Lak province also show a high proportion of molecular markers for artemisinin and piperaquine resistance and TES 2019 indicates late treatment failure. after PCR correction were 34% and 68% respectively in Gia Lai and Dak Lak [19].

4.3. Evaluation of the susceptibility of *P. falciparum* to antimalarial drugs in an *in vitro* trial in Gia Lai

The mean IC50 inhibitory concentration of AS was IC50 was 3.06 ± 3.10 nmol/L, higher than the results of 2004 study at Bu Gia Map Binh Phuoc, the average IC50 of AS was 1.3 ± 0. 6 nmol/L [27], also much higher than the multicenter study in western Cambodia in 2001-2007 on 495 samples with AS drug [83]. Compared to the study in Gabon Africa, the average IC50 was 2.08 nmol/L (n=34) [55]. Compared with the study in France [41], the average IC50 is 1.1 nmol/L, this result is much lower than the result of our study. A study in Thailand on P. falciparum patients collected from 2016-2017, the average IC50 value was 3.06 ± 3.10 nmol/L [67] This result is similar to the result in Gia Lai. 2016-2017.

The mean IC50 inhibitory concentration of DHA was 2.95 ± 2.19 nmol/L. This result is higher than the study in Cameroon in 1999 with an average IC50 of 1.11 nmol/L [92]. In a total of studies in France [41], the average IC50 of DHA was 1.3 nmol/L [41]. In a 2008 Kenyan study, the mean IC50 inhibitory concentration of DHA for 115 P. falciparum isolates was 2.0 ± 1.0 nmol/L [80]. In a study in Thailand in 2017, the mean IC50 inhibitory concentration of DHA for 113 P. falciparum isolates was 2.1 ± 1.2 nmol/L [67].

In addition, piperaquin phosphate (PPQ) acts as a partner drug in the DHA-PPQ combination tablet. Average IC50 value obtained 43.5 ± 24.9 nmol/L. In a study in France, a total of 181 *P. falciparum* patients [41], the average IC50 of PPQ was 66.8 nmol/L, this result is higher than the study results in Gia Lai. In a

study at the border between Thailand and Cambodia, Myanmar in 2016 on patients infected with *P. falciparum* alone, the mean IC50 inhibitory concentration of PPQ was 16.7± 6.3 nmol/L [68], Study at In Thailand in 2017 in patients infected with *P. falciparum* alone, the mean IC50 inhibitory concentration of PPQ was 18.4 ± 8.4 nmol/L [67]. In a Kenyan study in 2008, the mean IC50 inhibitory concentration of PPQ for 115 P. falciparum isolates was 42 ± 10 nmol/L [80].

CONCLUSION

1. Description of some epidemiological characteristics of K13 gene mutation of *Plasmodium falciparum* in 5 provinces of Binh Phuoc, Gia Lai, Khanh Hoa, Ninh Thuan, Quang Tri with severe malaria in 2016 - 2018

The rate of K13 gene mutation in 5 provinces was 46.23% (135/292). In which, K13 mutation in Binh Phuoc is 97.44% (38/39), Gia Lai 62.03% (67/108), Khanh Hoa 32.69% (17/52), Ninh Thuan 6.82%. (3/44), Quang Tri 20.41% (10/49); All K13 mutations in Binh Phuoc, Ninh Thuan and Quang Tri are of C580Y type, accounting for 97.44% of Binh Phuoc respectively; Ninh Thuan 6.82% and Quang Tri 20.41%; In Gia Lai, the K13 mutation type is more diverse, in which the C580Y type accounts for 45.37% and the P553L type is 5.56%, which are two types of mutations determining artemisinin resistance and the related mutation C496F is 4.63% in addition. There are also 2 mutations of unknown role, K503N and Y511H, accounting for 6.48%; In Khanh Hoa, there are 2 mutations determining resistance: C580Y is 5.77%, P553L type is 21.15% and three other types H384Q , G638E, G639D are 5.77%.

2. Evaluation of the response of the malaria parasite *Plasmodium falciparum* to the drug dihydroartemisinin - piperaquin phosphate at the study sites

- Clinical and complete parasitological response (ACPR) of *P. falciparum* to DHA-PPQ in Ninh Thuan and Quang Tri were 100%, Khanh Hoa province 97.4%, Gia Lai 97.8%. Binh Phuoc is 81.8% (< 90%); Given the failure rate in Binh Phuoc, it is necessary to find an alternative ACT combination.

- The survival rate of clonal parasites after DHA-PPQ treatment on day D3 at the monitoring points were: Binh Phuoc 41%, Gia Lai 14.6%, Khanh Hoa 18.6%, Ninh Thuan 0% and Quang Treat 0%.
- All 31 patients with D3 parasites have mutations in the K13 gene. All of these mutations are classified as defining artemisinin resistance.

3. *In vitro* susceptibility of *P. falciparum* isolates to antimalarial drugs in Gia Lai province

- The rate of parasites developing into schizonts in control wells above 10% was 42/54 samples (77.7%).
- The average IC50 of AS for *P. falciparum* is 3.06 ± 3.10 nmol/L. The mean IC50 of DHA for *P. falciparum* was IC50 of 2.95 ± 2.19 nmol/L. The mean IC50 value of PPQ is 43.5 ± 24.9 nmol/L.
- Average IC50 of AS and DHA in the group with K13 gene mutation was higher than in the group without mutation, this difference was statistically significant ($p < 0.05$).

RECOMMENDATIONS

1. The role of K13 gene mutation as an early warning and an additional factor for the valid definition of artemisinin resistance Tracing the spread of drug resistance between some regions, Futher monitor should be continued to supervise signal Molecular on *P. falciparum* populations to warn;
2. Once the K13 gene mutation associated with artemisinin-resistant *P. falciparum* has been identified, The study of distribution of K13 gene mutations should be in all provinces with severe malaria and suspected artemisinin resistance;
3. It is necessary to closely monitor and manage the treatment process, especially for *P. falciparum* malaria patients, promptly detect cases of early failure to replace antimalarial drugs,and simultaneouslyHaving plan to control parasite resitance strategically.
4. It is recommended to study and analyze molecular markers Pfplasmepsine 2/3 and PfExoE415G related to resistance to piperaquin phosphate component (PPQ) in DHA-PPQ combination tablets, in order to thoroughly assess the status of DHA-PPQ drug resistance. PPQ at that time.