

SERIOUS ANTIMALARIA RESISTANCE, GENETIC MARKERS OF KELCH 13, PLASMEPSINE 2 CNV ASSOCIATED WITH DIHYDROARTEMISININE - PIPERAQUINE RESISTANCE IN *Plasmodium falciparum* POPULATION IN MALARIA ENDEMIC ZONE OF DAKLAK PROVINCE, 2019 - 2020

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Summary

Backgrounds: Dihydroartemisinin - piperazine is a current frontline drug recommended in global by WHO for the treatment of *Plasmodium falciparum* malaria (WHO, 2015), but is now failing in Vietnam provinces where border Cambodia, and has emerged and spread. The purpose of this study was to evaluate efficacy and molecular markers of dihydroartemisinin - piperazine failures in Daklak province. **Methods:** A study design of non - randomized controlled study design for the 42 day - course follow - up in vivo test, and the molecular markers analysis. **Findings:** The data showed that adequate clinical and parasitological response was sharply declined of 12,1%, late clinical failure of 51.5%, late parasitological failure of 36.4%, proportion of positive parasitemia at D3 is 37%, slope half - life of 5.36 hrs, and progressive parasite clearance PC50, PC75, PC 90, PC95, and PC99 were 13.24; 19.29; 25.69; 29.97 and 39.15 hrs, respectively. Molecular markers of C580Y Kelch mutation observed 100% (50/50) in the patients, increased of Plasmepsine 2 CNV of 72% (36/50), and both K13 and Plasmepsine 2 of 72% (36/50). **Conclusions:** The DHA - PPQ efficacy severely decreased of 12.1%, overall treatment failure of 87.9% with the prominent C580Y mutant plus increased Plasmepsine 2 copy number variation in delayed asexual *P. falciparum* parasite clearance. These obvious data need to urgently change antimalarial policy in DHA - PPQ resistance zones.

Key words: *Plasmodium falciparum*, K13 propeller, Plasmepsine 2 copy number variation (CNV).

INTRODUCTION

Artemisinin - resistant *Plasmodium falciparum* threatens to the malaria elimination process in the Greater

Mekong Subregion (GMS)^[10]. K13 - propeller mutation and the increased copy number variations (CNVs) in Plasmepsine 2 were considered as markers associated with artemisinin - resistance and piperazine - resistance, respectively, and had a role in the extended period of asexual development^[1], which consequently caused delayed parasite clearance after treatment with ACTs^[2,3]. These markers were detected in several countries in GMS. Binh-phuoc, and many provinces in Central Highlands already have reduced sensitivity, treatment failure and resistance to dihydroartemisinin-piperazine (DHA - PPQ), especially

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in Daknong, Quangnam, Khanhhoa and Gialai. Krongnang district in Daklak province is one of the hot spots in terms of malaria cases in Central Highlands, and border Daknong, Khanhhoa, Gialai, Phuyen, then, are there in vivo resistance to DHA - PPQ and resistance markers in these provinces? Therefore, this study was conducted to evaluate DHA - PPQ efficacy in *P. falciparum* treatment and molecular markers (K13 - propeller mutation and Plasmepsine 2) in two *P. falciparum* populations.

SUBJECTS AND METHODS

Study sites and duration: Eadak, Dlie Ya and Ea Puk communes in Krong Nang district, Daklak province. From February 2019 to February 2020.

Study design: Non - randomized controlled study design for the 42 day - course follow - up according to the WHO template protocol for therapeutic efficacy studies (TES) (WHO, 2019).

Molecular markers analysis at Molecular biology unit of Pasteur Institute, Cambodia.

Study population: Inclusion criteria: patients with age between 3 and 70 years, mono - infection with *P. falciparum*, parasitaemia between 1000 and 100000/ μ l of asexual forms, presence of tympanic temperature $\geq 37,5^{\circ}\text{C}$, no prior antimalarial drugs, informed consent from patient or from a parent or guardian in the case of children.

Exclusion criteria: patients with age under 3 years or more than 70 years, pregnancy or breastfeeding, patients with mental health disorders, epilepsy, severe vomiting or diarrhea, or inability to absorb oral medications, complicated malaria or co - infected diseases, mixed - infection with another Plasmodium species, patients with prior antimalarial drugs, women in child - bearing age have to take pregnancy test.

Study sample size: The proportion of DHA - PPQ treatment failure in a previous study in Binh Phuoc was 15%. Therefore, 15% has been chosen as the estimated therapeutic failure rate of the drug, with a confidence level of 95% and with a precision around the estimate of 10%. The estimated minimum sample size was $n = 50$.

Study assessment: All patients who meet the basic enrolment criteria will be evaluated for clinical information, malaria parasite density, parasite clearance measurement, molecular biological analysis, K13 mutation (artemisinin -

resistance) identification, increased CNV in Plasmepsine 2 (piperazine-resistance) detection by RT - PCR with Synb green staining.

Statistical analysis: *In vivo* software (WHO 2015, version 2017) will be used for data management and analysis. The proportion of K13 - propeller and Plasmepsine 2 mutation will be calculated per total analyzed sample.

RESULTS

Baselines of study patient's characteristics

Table 1. Characteristics of study population

Characteristics	Krongnang - Daklak	
	Number	%
Gender	50	
Male	47	94.0
Female	3	6.0
Mean Age (years)	32.1 (7.9)	
Min - Max	19 - 51	
< 5	0	0
$\geq 5 - < 15$	0	0
≥ 15	50	100
Mean weight (kg)	56.9 (5.6)	
Min - Max	43 - 72	

In all 50 patients who were mono - infected with *P. falciparum* in Krongnang, Daklak, there was a male predominance with 47 cases (94%), while there were only 3 female patients (6%). The mean age was 32.1 years and all patients were more than 15 years old (100%). The mean weight was 56.9 kg.

Table 2. Clinical manifestations, spleen examination and *P. falciparum* parasite density

Characteristics	Daklak (n = 50)
Temperature	Mean \pm SD (Min - Max)
Mean temperature ($^{\circ}\text{C}$)	38.5 \pm 0.6 (36.2 - 39.6)
Spleen feature	
Splenomegaly \geq stage 2	3 (6.0)
History of splenectomy	0 (0)
Parasite density before treatment	
Mean parasite density	14.263
Min - Max	(828 - 98.102)
Hematological tests	
Haemoglobine (g/dL)	11,4 (9.5 - 11.2)
Haematocrite (%)	37.2 (39.5 - 40.2)

The mean temperature of all patients was 38.5 \pm 0.6 $^{\circ}\text{C}$, spleen examination detected only 3 patients with splenomegaly greater or at stage 2 (%), there was no patient with history of splenectomy. The mean parasite

density at D₀ (before treatment) was 14.263/μl. The mean haemoglobin (Hb) was 11.4g/dL (9.5 - 11.2) and mean haematocrit (Hct) was 37.2% (39.5 - 40.2), these two figures were in normal range.

The efficacy of DHA - PPQ in treatment of *P. falciparum*

Table 3. The efficacy of treatment of *P. falciparum* at study sites

	Treatment outcomes	Unadjusted PCR		Adjusted PCR	
		Number	%	Number	%
Efficacy outcomes	Early treatment failure (ETF)	0	0	0	0
	Late clinical failure (LCF)	17	41.5	17	51.5
	Late parasitological failure (LPF)	20	48.8	12	36.4
	Adequate clinical and parasitological response (ACPR)	4	9.7	4	12.1
	Total analysis (per protocol)	41		33	
Withdrawal and loss to follow - up	9	18.0	17	34	
Total	50		50		

In 50 patients who were followed - up to 42 days, the number of Adequate clinical and parasitological response (ACPR) to DHA - PPQ after PCR was 4 (12.1%), 17 patients (51.5%) had late clinical failure (LTF) and 12 patients (36.4%) had late parasitological failure (LPF), there was no late treatment failure. The number of withdrawing and loss to follow - up patients was 17 (34%).

Table 4. Treatment efficacy and asexual parasite detection after treatment with DHA - PPQ

Analytic indicators	Daklak (n = 50)			
	Number	%	CI95%	
Parasite clearance before D3 after treatment with DHA - PPQ	33	63.0		
Asexual parasite detection at D3 (or H72)	17	37.0	23.2%	52.5%

The proportion of asexual parasite detection at D3 after treatment was 37%.

Table 5. Indicators of *P. falciparum* parasite clearance in follow - up

In vivo cases	Slope half - life	Parasite clearance process				
		PC50	PC75	PC90	PC95	PC99
50	5.36 hours	13.24 hours	19.29 hours	25.69 hours	29.97 hours	39.15 hours

Detailed analysis indicated that slope half - life was 5.36 hours. The 50% - clearance of parasite (PC50) was 13.24 hours. PC75, PC90, PC 95 and PC 99 were 19.29 hours, 25.69 hours, 29.97 hours and 39.15 hours, respectively.

Identification of K13 mutation and increased Plasmeppsine 2 CNV

Table 6. Markers analysis of K13 propeller and plasmepsine 2 CNV

Study site	K13 mutation and increased Plasmeppsine 2 CNV (> 1.5 copies)			
	Sample size	K13 mutation	Increased Plasmeppsine 2 CNV (> 1.5 copies)	K13 + Plasmeppsine 2
Daklak (n = 50)	50	C580Y 50 (100%)	36 (72%)	36 (72%)
Association →		Artemisinin resistance	Piperaquine resistance	Artemisinin and Piperaquine resistance

Molecular analysis of *P. falciparum* in 50 patients who attended to the study in Krongnang district, Daklak province, showed that 50 cases (100%) had C580Y mutation, associated with resistance to artemisinin.

The number of increased Plasmeppsine 2 CNV (1.5 copies) in *P. falciparum* population was 36 (72%), it was considered as a marker of resistance to piperaquin phosphate and the number of cases which had both those two makers was 36 (72%), which means resistance to two molecules in DHA - PPQ.

Table 7. Comparison between mutation type and WHO K13 mutation classification

WHO classification (WHO, 2018)			
K13 mutation	Classification	K13 mutation	Classification
P441L	Associated ⁽¹⁾	F637I	Associated ⁽¹⁾
G449A	Associated ⁽¹⁾	Y493H	Confirmed ⁽²⁾
C469F	Associated ⁽¹⁾	R539T	Confirmed ⁽²⁾
A481V	Associated ⁽¹⁾	I543T	Confirmed ⁽²⁾
V568G	Associated ⁽¹⁾	P553L	Confirmed ⁽²⁾
P527H	Associated ⁽¹⁾	R561H	Confirmed ⁽²⁾
P574L	Associated ⁽¹⁾	C580Y	Confirmed ⁽²⁾
A675V	Associated ⁽¹⁾	N458Y	Confirmed ⁽²⁾
G538V	Associated ⁽¹⁾	F446I	Confirmed ⁽²⁾
N537I	Associated ⁽¹⁾	M476I	Confirmed ⁽²⁾

⁽¹⁾Associated is a mutation classification which associate to *P. falciparum* resistance

⁽²⁾Confirmed is a mutation classification which confirm *P. falciparum* resistance

DISCUSSION

Baseline data of clinico - laboratory profile of *P. falciparum* malaria patients

In the total of 50 cases of uncomplicated falciparum malaria, male gender is 47 cases (94%) and female 3 cases (6%). Average age of 32.1 and all of them are adult, average weight of 56.9kg. Average body temperature of $38.5 \pm 0.6^{\circ}\text{C}$, and 6.0% (3/50) of cases have spleen enlarge. Asexual mean geometric malaria parasite of *P. falciparum* at day D₀ is 14,263/ μl , and haematological parameters in study group is in normal range, especially is haemoglobine of 11.4g/dL (9.5 - 11.2) and haematocrite percentage of 37.12% (39.5 - 40.2), and these data are similar to other therapeutic efficacy studies in Daknong, Quangtri provinces (Vietnam), and Cambodia, and Myanmar sentinel sites.

Efficacy of DHA - PPQ in the treatment for uncomplicated falciparum malaria

Total of 41/50 cases of *P. falciparum* were followed - up to day 42 showed that PCR adjusted - adequate clinical and parasitological response (ACPR) is 12.1% (4/41), late clinical failure (LCF) of 51.5% (17/41), late parasitological failure (LPF) of 36.4% (12/41), and no early treatment failure (ETF). After the DHA - PPQ 3 - day course dose treatment, the day D₃ positive of asexual parasite of 37.0% (CI95%: 23.2 - 52.5). This data evoked that DHA - PPQ efficacy is seriously reducing, and cure rate only 12.1%, total treatment failure of 87.9%. Compared of ACPR to other border - line in Daknong province (border

Cambodia) and Quangtri province (border Lao PCR) were 88.1% and 100%, respectively. In particular, data in Daknong with LCF of 9.5%, LPF of 2.4% (n = 46), and D₃ positive was 41.7%. According to WHO terminology that proportion of D₃ > 10% as indirect marker of partial resistance of artemisinin and its derivatives. Hence, out of TES data and positive D₃ > 10%, WHO - validated molecular markers analysis will strongly confirm the artemisinin and partner drugs status. On the other hand, slope half - life is 5.36, and detailed parasite clearance time of parasite biomass gradually PC50, PC75, PC90, PC95, and PC99 are 13.24; 19.2; 25.69; 29.97, and 39.15, respectively.

These data are similar of other confirmed TES in malaria endemic areas in Central Highlands and Binhphuoc province of Vietnam^[1,2]. Proportion of ACPR are from 94.7 - 100% and D₃ positive of *P. falciparum* sexual form were still high, such as in Binhphuoc, this parameter increasing step by step, year by year, from 15% to 22%, 30%, 36%, 36.8% in 2010 - 2015; in Quangnam with D₃ proportion of 29.2%, Khanhhoa of 17.4%, Gialai was from 11% to 23%; 26.4% in 2010 - 2014. Compared to other sentinel sites of Greater Mekong Subregion countries, this proportion is little lower, especially in Pailin, Rattanakiri, Battambang of Cambodia have ACPR of 90 - 95% with D₃ positive in 2008 - 2012 period are 26%, 33%, 45%, 54%, respectively or this proportion is higher than Jingyang's D₃ positive in China (D₃ of 14%), or in Champassack of Lao PDR is 22% in 2012, or in the Kawthaung, Mon of Myanmar is from 14% up to 19%, 23% (2011 - 2013), or in the Tak, Maehongson, Kanchanaburi of Thailand^[10] with D₃ positive is from 9% to 14%, 17%, 25% and 48% in 2009 - 2014. All these results showed that not only DHA - PPQ resistance is in - country spreading out, but also occurred in GMS countries.

Molecular markers of Kelch13 and Plasmepsine 2 copy number variation

Analysis of *P. falciparum* isolates by molecular sequencing of K13 propeller mutants (for dihydroartemisinin - DHA resistance) and Plasmepsine 2 CNV (for piperazine - PPQ resistance), data showed that all of 50 (100%) isolates have C580Y genotype involved to artemisinin resistance, and increased Plasmepsine 2 (> 1.5 copy) on *P. falciparum* population is 72% (36 isolates) as PPQ resistance. The number of samples have K13 plus Plasmepsine 2 markers is 72%.

CONCLUSIONS

Efficacy of DHA - PPQ in the treatment for uncomplicated *P. falciparum* with ACPR of 12.1%, LCF of 51.5% and LPF of 36.4%. The positive asexual parasite on D₃ was 37.0%.

Type of K13 mutant of C580Y was 100% in all isolates and Plasmepsin 2 CNV was 72%, especially in both resistant markers in *P. falciparum* population was 72%. Both these data showed that the DHA - PPQ efficacy was reduced seriously and need to change antimalarial drug policy in this area by new ACTs.

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