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(RESEARCH ARTICLE)

Serious antimalaria resistance, genetic markers of Kelch 13, plasmepsine 2 CNV associated with dihydroartemisinine-piperaquine phosphate resistance in *Plasmodium falciparum* population in malaria hyperendemic zone of Dak Lak Province (2019-2020)

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Abstract

Dihydroartemisinin-piperaquine (DHA-PPQ) 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's provinces where border Cambodia, and has emerged and spread. The purpose of this study was to evaluate the efficacy and molecular markers of DHA-PPQ failures in Dak Lak 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 to 12,1%, the proportion of late clinical failure was 51.5%, and 36.4% of patients had late parasitological. The proportion of positive parasitemia at D3 was 37%, the slope half-life was 5.36 hrs, and the progressive parasite clearance (PC) PC50, PC75, PC 90, PC95, and PC99 were 13.24; 19.29; 25.69; 29.97 and 39.15 hours, respectively. Molecular markers of C580Y Kelch mutation were observed in 100% (50/50) of the patients, the increased of Plasmepsine 2 copy number variation (CNV) was 72% (36/50), and the proportion of patients who had both K13 and increased Plasmepsine 2 CNV was 72% (36/50). Conclusions: The DHA-PPQ efficacy severely decreased to 12.1%, overall treatment failure was 87.9% with the prominent C580Y mutant plus increased Plasmepsine 2 CNV in delayed asexual *P. falciparum* parasite clearance. These obvious data suggest the urgency to change antimalarial policy in DHA-PPQ resistance zones, especially in Dak Lak province.

Keywords: *Plasmodium falciparum;* K13 propeller; Plasmepsine 2 copy number variation (CNV).

1. Introduction

Artemisinin-resistant *Plasmodium falciparum* threatens to the malaria elimination process in the Greater Mekong Subregion (GMS). K13-propeller mutation and the increased copy number variations (CNVs) in Plasmepsine 2 were considered as markers associated with artemisinine-resistance and piperaquine-resistance, respectively, and had a role in the extended period of asexual development, which consequently caused delayed parasite clearance after treatment with artemisinine-based combination therapies (ACTs). 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 dihydroarteminsinin-piperaquin (DHA-PPQ), especially in Dak Nong, Quang Nam, Khanh Hoa and Gia Lai. Krong Nang district in Dak Lak province is one of the hot spots in terms of malaria cases in Central Highlands, and border Dak Nong,

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Khanh Hoa, Gia Lai, Phu Yen, 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.

2. Material and methods

2.1. Study sites and duration

Ea Dak, Dlie Ya and Ea Puk communes in Krong Nang district, Dak Lak province. From February 2019 to February 2020.

2.2. 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.

2.3. Study population

Inclusion criteria: patients with age between 3 and 70 years, mono-infection with *P. falciparum,* parasitaemia between 1000 and 100000/µl of asexual forms, presence of tympanic temperature ≥ 37.5^{o}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.

2.4. 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.

2.5. 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 (piperaquine-resistance) detection by RT-PCR with Synbgreen staining.

2.6. Statistical analysis

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

3. Results

3.1. Baselines of study patient's characteristics

In all 50 patients who were mono-infected with *P. falciparum* in Krong Nang, Dak Lak, 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.

The mean temperature of all patients was 38.5 ± 0.6^{O}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 11.4 g/dL (9.5 – 11.2) and mean haematocrit (Hct) was 37.2% (39.5-40.2), these two figures were in normal range.

Table 1 Characteristics of study population

Characteristics	Krong Nang - Dak Lak	
	Number	**%**
Gender Male Female	50 47 3	 94,0 6,0
Mean Age (years) Min - Max < 5 ≥ 5 - < 15 ≥ 15	32,1 (7,9) 19-51 0 0 50	 0 0 100
Mean weight (kg) Min - Max	56,9 (5,6) 43 - 72	

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

Characteristics	**Dak Lak (n = 50)** Mean ± SD (Min-Max)
Temperature (^{0}C)	38,5 ± 0,6 (36,2 - 39,6)
Spleen feature Splenomegaly ≥ level 2 History of spleenectomy	 3 *(6,0)* 0 *(0)*
Parasite density before treatment Mean parasite density Min-Max	 14.263 (828 - 98.102)
Hematological tests Haemoglobin (g/dL) Haematocrit (%)	 11,4 (9,5 -11,2) 37,2 (39,5 - 40,2)

3.2. 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 33/50 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 early 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

Analystic indicators	**Dak Lak (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.

3.3. Identification of K13 mutation and increased Plasmepsine 2 CNV

Table 6 Markers analysis of K13 propeller and plasmepsine 2 CNV

Study site	**K13 mutation and increased Plasmepsine 2 CNV (> 1,5 copies)**			
	Sample size	**K13 mutation**	**Increased Plasmepsine 2 CNV (> 1,5 copies)**	**K13 + Plasmepsine 2**
Dak Lak (n = 50)	50	C580Y 50 (100%)	36 (72%)	36 (72%)
Significant association →		Artemisinine resistance	Piperaquine resistance	Artemisinine and Piperaquine resistance

Molecular analysis of *P. falciparum* in 50 patients who attended to the study in Krong Nang district, Dak Lak province, showed that 50 cases (100%) had C580Y mutation, associated with resistance to artemisinine;

The number of increased Plasmepsine 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 associated to *P. falciparum* resistance
[(2)]Confirmed is a mutation classification which confirmed *P. falciparum* resistance

4. Discussion

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

In the total of 50 cases of uncomplicated *P. falciparum* malaria, there were 47 male (94%) and 3 female (6%). The average age was 32.1 years and all of the patients were adult, the average weight was 56.9kg. The average body temperature was 38.5 ± 0.6^0C, and 6.0% (3/50) of patients had spleen enlargement. Asexual mean geometric malaria parasite density of *P. falciparum* at day D_0 was 14,263/µl, and hematological parameters was in normal range, specifically, the mean hemoglobin was 11.4 g/dL (9,5-11,2); the mean haematocrit was 37.12% (39,5-40,2), and these data were similar to other therapeutic efficacy studies in Dak Nong, Quang Tri provinces (Vietnam), and Cambodia, and Myanmar sentinel sites.

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

Total of 33/50 cases of *P. falciparum*-infected patients were followed-up to day 42 the number of Adequate clinical and parasitological response (ACPR) to DHA-PPQ after PCR-adjusted 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 early treatment failure (ETF). After 3-day course of treatment with DHA-PPQ, the proportion of asexual parasite detection at D3 was 37% (CI95%: 23.2-52.5). The data of DHA-PPQ efficacy in this study was unrelated to previous studies in Vietnam, such as other TESs in malaria endemic areas in Central Highlands and Binh Phuoc province when the proportion of ACPR was from 94.7-100%. From 2014 to 2016, Huynh Hong Quang and his team conducted a study in several communes in Binh Thuan and Dak Lak, the results showed that the proportion of ACPR was 100% and there was no D3 positive of asexual form. Some studies in Binh Phuoc (Tran Tinh Hien *et al.,* 2014), Dak Nong, Gia Lai, and Quang Nam (Huynh Hong Quang *et al.,* 2014) showed similar results with from 91.2 to 100% of ACPR, but the proportion of D3 positive of asexual form was from14.7 to 44%. In Binh Phuoc, the proportion of D3 positive of asexual form was increasing step by step, year by year, from 15% to 22%, 30%, 36%, 36.8% in the period of 2010 and 2015. In Gia Lai, this figure increased from 11% in 2010 to 23% in 2014. This meant that the DHA-PPQ efficacy in this study was seriously reduced with the cure rate of 12.1%, treatment failure of 87.9%).

In some sentinel sites in Greater Mekong Subregion countries, particularly in Pailin, Rattanakiri, Battambang provinces in Cambodia, the proportion of ACPR ranged from 90% to 95% and D3 positive percentages in 2008-2012 period were 26%, 33% and 54%, respectively. This proportion was higher than in Jingyang, China (14%), or in Champassack, Lao PDR (22% in 2012), or in the Kawthaung, Mon of Myanmar (from 14% to 23% in 2011-2013), or in Tak, Maehongson, Kanchanaburi, Thailand (from 9% to 14%, 17%, 25% and 48% in 2009-2014). In addition, recent studies in Greater Mekong Subregion showed that the ACPR was decreasing in many provinces in Cambodia, Myanma and Laos. According to WHO terminology, the proportion of D3 > 10% is an indirect marker of partial resistance of artemisinin and its

derivatives. All of these results showed that not only DHA-PPQ resistance was in-country spreading out (D3 positive of asexual form proportion was 37%), but also occurred in GMS countries. Consequently, the DHA-PPQ efficacy was significantly reduced (ACPR proportion in this study was only 12.1%).

According to WHO report (2019) on artemisinin resistance and artemisinin-based combination therapy efficacy The analysis of DHA-PPQ included 130 studies conducted in 21 countries. Studies of DHA-PPQ demonstrated an overall efficacy of 94.5%. In the WHO African Region, 21 studies conducted in Angola, Congo, Gambia, Guinea Bissau, Kenya, Malawi, Nigeria, Senegal, Sierra Leone and Zambia demonstrated an overall efficacy of 99.3%. In the WHO Eastern Mediterranean Region, eight studies conducted in Pakistan, Somalia and Sudan showed a treatment efficacy of 99.3%. In the WHO South-East Asia Region, 28 studies conducted in Indonesia, Myanmar and Thailand showed a treatment efficacy of 99%. In the WHO Western Pacifc Region, 74 studies were conducted in Cambodia, China, Lao People's Democratic Republic, Papua New Guinea and Viet Nam. The overall treatment efficacy was 90.7%. Treatment failure rates greater than 10% were observed in 19 studies from Cambodia, Laos and Viet Nam. In Cambodia, treatment failure rates exceeded 10% in 13 of the 27 studies conducted; the maximum treatment failure rate was 62.5% in 2014. This evidence has prompted discussions of a change in Viet Nam's current treatment policy in areas where DHA-PPQ is failing.

4.3. Molecular markers of Kelch13 and Plasmepsine 2 CNV

Analysis of *P. falciparum* isolates by molecular sequencing of K13 propeller mutants (for dihydroartemisinin-DHA resistance) and Plasmepsine 2 CNV (for pieraquine-PPQ resistance), data showed that all of 50 (100%) isolates had C580Y genotype involved to DHA resistance, and 36 (72%) isolates had an increased Plasmepsine 2 (>1,5 copy) as PPQ resistance. The number of samples which had both K13 and Plasmepsine 2 markers is 72%.

Comparing analytical data on the molecular mutation with other authors in recent times on a large scale in many provinces with *P. falciparum* populations in malaria endemic areas in Vietnam from 2009-2016, the propeller K13 mutant is known as a molecular marker associated with artemisinin resistance recognized by WHO and has been sequenced on 1,060 *P. falciparum* isolates of 3 malaria hotspots in Vietnam. The frequency of K13 mutation ranged from 29% (222/767), 6% (11/188) and 43% (45/105) in Binh Phuoc, Ninh Thuan and Gia Lai provinces, respectively. The geographical distribution based on these molecular data showed that the Central Highlands provinces such as Gia Lai, Kon Tum, Dak Lak and Dak Nong and the South province such as Binh Phuoc, which has a border with Cambodia; had the same mutations and C580Y was the most common. While in some provinces which border Laos such as Quang Tri province, P574L was the most common (WHO, 2018). In addition, data on assessment of the molecular resistance in Champasack, Laos in 2013 showed that the C580Y mutation related to drug resistance and was the dominant mutation in severe malaria regions of the Greater Mekong Subregion countries (WHO, 2016).

Artemisinin resistance has been confirmed in Cambodia, Laos and Viet Nam through several studies conducted between 2001 and 2018. Between 2010 and 2018, eight *PfKelch13* mutations were identifed in Cambodia and Laos. C580Y was the most frequent, with about 71.7% of the genotypes carrying this mutation. In Viet Nam, six *PfKelch13* mutations were identifed, and C580Y was also the most predominant, appearing on an average of 33.3% of the genotypes. The *PfKelch13* mutation C580Y has been identifed twice in Papua New Guinea. No validated molecular markers of artemisinin resistance were found in studies conducted in Malaysia, the Philippines, Solomon Islands or Vanuatu.

Further more, increased plasmepsine CNV which is an indicator of reduced-sensitivity and/or resistance to PPQ, has been evaluatated to explain clearly the resistance to DHA-PPQ combination in recent two years in some malaria endemic ares in the Greater Mekong Subregion countries such as Cambodia, Thailand, Myanmar and Vietnam with valuable molecular data for early identification of resistance.

5. Conclusion

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

C580Y type of K13 mutant was 100% in all isolates, the proportion of increased Plasmepsin 2 CNV was 72%, and the percentage of isolates which had both K13 and Plasmepsine 2 markers was 72%.

Both of these data showed that the DHA-PPQ efficacy was reduced seriously and suggested an urgent change in antimalarial drug policy in Dak Lak province.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declared that they have no conflicts of interest.

Statement of informed consent

The research protocol was reviewed by the Institutional Review Board of the Institute of Malariology, Parasitology and Entomology Quy Nhon.

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