MOLECULAR ANALYSIS OF *PFMDR1* GENE IN *PLASMODIUM FALCIPARUM* IN THAI-CAMBODIA BORDER

Prapatchaya Pa-onta, Naowarat Kanchanakhan*

College of Public Health Sciences, Chulalongkorn University, Bangkok, 10330, Thailand

ABSTRACT:

Background: Drug resistant *Plasmodium falciparum* is a major problem for malaria control. Policy makers currently depend on *in vivo* and *in vitro* test to adjust antimalarial regimens for malaria treatment guideline.

Methods: These two methods are required expertise for interpretation and time consuming. Therefore, the alternative reliable molecular markers of antimalarial resistance could also play an important role in the surveillance of drug efficacy. *Pfmdr1* gene has been shown to be a reliable marker of resistance for *P. falciparum* related to artesunate and mefloquine combination therapy. The propose of this study are to investigate the prevalence of *P. falciparum* multidrug resistance by determined *Pfmdr1* point mutations at codon N86Y, Y184F, S1034C by PCR-RFLP and investigated *Pfmdr1* copy number by using real-time quantitative PCR with TaqMan and compared to efficacy of ACTs (Artemisinine Combination Therapies).

Results: Seventy-three infected blood samples were collected from the therapeutic efficacy of ACTs project tested in 2 provinces in malaria endemic areas of Thailand which are Chantaburi and Trat. The results showed that in Trat province exhibited the higher percentage of *P. falciparum* with three or more copies of *Pfmdr1* than in Chantaburi (27.27% and 0%, respectively). The mean of *Pfmdr1* copy number in *P. falciparum* collected from Trat and Chantaburi provinces were 2.2 and 1.5 respectively. In contrast, there was no mutation in *Pfmdr1* gene in *P. falciparum* from Trat whereas in Chantaburi found point mutation in N86Y (20%, n=15), S1034C (28.57%, n=7) and no mutation was found in Y184F both in *P. falciparum* collected from Trat and Chantaburi.

Conclusion: These results were correlated to the ACTs efficacy test. *Pfmdr1* mutation and copy number may be used as a high throughput tool to investigate the role of drug resistance of malaria parasites in laboratory studies or large scale epidemiological surveys.

Keywords: Real-time PCR; *Pfmdr1*; *Plasmodium falciparum*; Drug resistance

DOI: 10.14456/jhr.2017.92 *Received: June 2016; Accepted: March 2017*

INTRODUCTION

Malaria is potentially infectious disease which is a major worldwide public health problem with appearance resistant of anti-malarial drugs, especially *Plasmodium falciparum* that develop to resist all classes of anti-malarial drugs including of the artemisinin derivatives. Furthermore, malaria is caused by protozoa parasite, *Plasmodium* species. There are five species of *Plasmodium* parasite can cause infectious malaria in human; *P. falciparum*, *P. malariae*, *P. ovale*, *P. vivax*, *and P. knowlesi*. The most severity is *P. falciparum* infection that causes life-threatening condition, cerebral malaria and this species also develop to resist all classes of anti-malarial drugs including the artemisinin. In human, the infection can occurred by intermediate hosts essentially female Anopheles mosquitoes. The incident of this disease frequently occurs more than 100 countries in tropical and subtropical area,

* *Correspondence to: Naowarat Kanchanakhan*
E-mail: Naowarat.K@Chula.ac.th

Cite this article as: Pa-onta P, Kanchanakhan N. Molecular analysis of *pfmdr1* gene in *Plasmodium falciparum* in Thai-Cambodia border. J Health Res. 2017; 31(Suppl.2): S251-9. DOI: 10.14456/jhr.2017.92

commonly in Africa, India, Latin America, and South-East Asia [1]. In Thailand, malaria is potential infection disease in international border areas, Thai-Myanmar, Thai-Laos, Thai-Malaysia, and Thai-Cambodia such as Trat province and Chanthaburi province.

Nowadays the most effective treatment for the malaria infection is artemisinin-based combination therapy (ACTs). In Thailand, artesunate-mefloquine is suggested by World Health Organization (WHO) that an assumption is the combination-drug partner can protect artemisinin resistance. However, recent documents indicate that artemisinnin resistance has already appeared along the international border of Thailand. The *Plasmodium* parasite becomes more resistant to these drugs especially *P. falciparum* species. A serious problem that limits the efficiency of malarial control program is the distribution of multidrug resistance to *P. falciparum* [2, 3].

The *P. falciparum* multidrug resistance gene (*Pfmdr1*) has been reported that it is related to alterative susceptibility to different anti-malarial drugs. This gene locates on chromosome 5 and encodes 12 transmembrance-domain proteins, which is called P- glycoprotein homologue 1 (Pgh1). This protein locates at digestive vacuole, where is the site of action of chloroquine and other quinoline-base antimalarial drug. However, the relation between *Pfmdr1* and artemisinin drugs resistance have remained difficult to understand. Resistance mechanisms providing this mainly implicate mutations and amplifications of the gene encoding target enzymes or transporters. A single genetic mutation in Pgh1 is not involved the resistance of chloroquine and mefloquine but Reeds and colleagues mentioned that the mutations in Pgh1 can lead to mefloquine, quinine and halofantrine resistance. The same mutations impacts parasite resistance towards chloroqiune in a strain-specific manner and the level of sensitivity unrelated to artemisinin [4-7]. This is important assumptions for the development of anti-malarial agents [8]. Furthermore, the amplification of *Pfmdr1* gene has been reported to relate with malaria multi-drugs resistant. There have been various reports using *Pfmdr1* copy number as a molecular tool for monitoring anti-malarial drugs efficiency [9, 10].

In this study, we investigate the prevalence of *P. falciparum* multidrug resistance by determining *Pfmdr1* copy number and *Pfmdr1* point mutations of *P. falciparum* endemic in Thai-Cambodia border, Trat and Chanthaburi provinces of Thailand.

METHODOLOGY

Samples collection from study sites

Total of 73 *P falciparum* infected blood samples were obtained from malaria patients attended at malaria clinics at Trat and Chantaburi provinces during 2006 to 2010. 49 samples were originally collected from Trat while 24 samples were received from Chantaburi province. After confirmation of *P. falciparum* infection by microscopic observation of thick Giemsa-stained blood films, the blood samples were spotted onto Whatman filter paper and then returned to perform DNA extraction in the laboratory. These samples were the samples from the patients participated in the ATCs (Artesunate+Mefloquine+Primaquine) treatment efficacy Project of Mrs Saowanit Vijaykadga (Ministry of Public Health, Thailand). The *in vivo* treatment followed the WHO guideline with all blood samples were collected in Day 0 before ACTs treatment. After ACTs treatment, the patients had been followed up for 42 days.

DNA extraction from field isolates of *P. falciparum*

Blood samples collected in the Whatman filter papers were incubated in PBS solution overnight at 4°C. After that the PBS solutions were replaced with 0.5% saponin in PBS and incubated at 4°C for 30 minutes. Then treated filter papers were transferred to hot 5% chelex-100 resin solutions (Bio-Rad Laboratories, CA) and incubated at 95°C for 10 minutes, vortex briefly between incubations. Supernatants were kept until used for parasite genotyping after centrifugation [11].

Detection of *Pfmdr1* polymorphisms by PCR-RFLP

Genomic DNA was extract using Chelex-resin (Biorad Co.Ltd., USA) according to the method of Plowe and colleague in 1995 [11]. Previously published and PCR-RFLP methods were employed to detect *Pfmdr1* at the codons 86, 184, and 1034 [12-14]. The primers and reaction conditions used were according to the previously described methods [12-14]. PCR were performed in a total volume of 25 µl with the following reaction mixture: 0.1 µM of each primer, 2.5 mM $MgCl_2$, 100 µM of each deoxynucleotide triphosphate, 1×PCR buffer (100 mM KCl, 20 mM Tris-HCl pH 8.0), 2 µl of genomic DNA. One cycle of 94°C for 2 min; 40 cycles of 94°C for 1 min; 45°C for 1 min and 72°C for 1 min; one cycle of 72°C for 5 min in a reaction.

Genomic DNA extracted from 3D7, K1 and PFK12 *P. falciparum* clones were used as positive

Table 1 Primer sequences

	Primers	Sequences
TaqMan	*Pfmdr1*-1F	AAACTTTAAAGCTTGAATATTTAAGAAGTGTTTT
	Pfmdr1-1R	GAACTCACTTGTTCTAAATAAAAATCTAAATCAGATCTTAAT
	Pfmdr1-probe	FAM-CAAGATGGACAATTTC-MGB
	Actin-1F	CCAGAAGCTTTATTCCAACCATCCT
	Actin-1R	CATTTTTTAATAGAGTTGAAAGTAGTTGTGTGGA
	Actin-probe	FAM-CCTGCTGCTTCTTTTC-MGB

Table 2 Enzyme incubation conditions and expected nucleic acid sizes for detection of amino acid mutation of *Pfmdr1*

Target residue	Primer	Restriction enzyme	Incubation condition	Expected size (bp)	
				Wild type	Mutation
N86Y	A2	*Afl III*	37°	560	232+328
	A4	*Apo I*		505	249+256
Y184F	A2	*Dra I*	37°	242+204	242+173
	A4				
S1034C	1034f	*Dde I*	37°	172+60	205+27
	1042r				

* The incubation reactions were performed overnight.

controls, which contain different types of mutations, whereas water was used as a negative control.

The primer sequences for all PCR reactions are described in [13]. Restriction sites were already present for the polymorphisms at codon 86 (*Apo* I and *Afl* III digested when the asparagine and tyrosine codons were presented respectively); for the 184 (*Dra* I digested when the phenylalanine codon was presented); and for 1034 (*Dde* I digested when the serine was presented) [13].

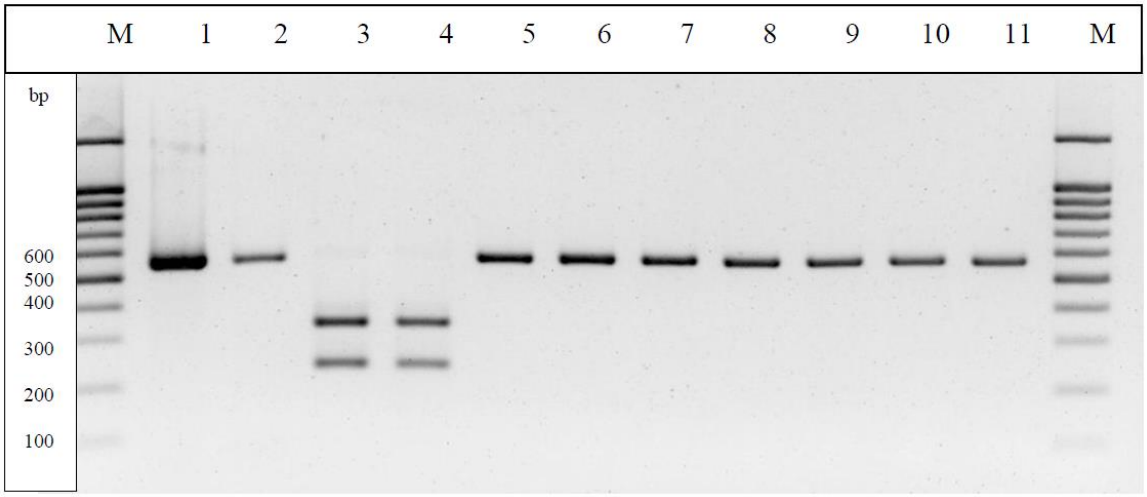
Unless otherwise stated, PCR products were analyzed by electrophoresis using 2% agarose gels, running in 1x TBE (Tris Borate EDTA buffer) containing ethidium bromide (0.5 µg/l in 1x TBE). Samples were loaded into wells after the addition of 1/5 volume of orange G dye (Sigma, U.K.) loading dye. DNA ladders were used as molecular weight markers and to aid in the size determination of PCR products. Electrophoresis was carries out at 100 V until the dye had electrophoresed about three fourth of length of the gel. Separated PCR products were visualized by UV transillumination (medium wavelength 302 nm).

Detection of gene copy number of *Pfmdr1* by TaqMan real-time PCR

Pfmdr1 copy number was assessed by TaqMan real-time PCR (Applied Biosystems, Warrington, UK). The primers and the probe are specific to a conserved region of *Pfmdr1, β-Actin*, and the designed sequences of these primers and probes were shown in Table 1. *Pfmdr1* copy number was analyzed according to the modified method developed by Price et al. [15]. Briefly, the amplification reactions was done as multiplex PCR in MicroAmp 48-well plates (Applied Biosystem) in a 25 µl reaction mixture containing TaqMan buffer pH 8.3 (8% glycerol, 0.625U DNA polymerase, 5.5 mmol/l $MgCl_2$, 300 µmol/l dNTP, 600 nmol/l passive reference dye 5-carboxy-Xrhodamine), 300 nmol/l of each forward and reverse primer, 100 nmol/l of each probe, and 5 µl of templates DNA. Fluorescence data was expressed as normalized reporter signal, calculated by dividing the amount of reporter signal by the passive reference signal. The detection threshold was set above the mean baseline value for fluorescence of the first 15 cycles, the threshold cycle (C_T) was done. So, that the reactions were performed for 40 cycles (Pre-Incubation at 95°C for 2 min, 1 cycle, amplification program: denature at 94°C 2 min, annealing at 45°C 40 sec, extension 72°C 1 min, melting 72°C 5 min and a final cooling step to 40°C). When the increase in reporter signal was first detected above baseline, the results were analyzed by a comparative C_T method based on the assumption that the target (*Pfmdr1*) and reference (*β-actin*) was amplified with the same efficiency with in an appropriate range of DNA concentrations. The comparative $\Delta\Delta C_T = C_TE - C_TB$, where C_TE denotes the experimental C_T and C_TB the baseline C_T. Every TaqMan run contained the reference DNA samples from 3D7 clones which contained only one copy numbers of *Pfmdr1*. All reactions were performed in triplicate and results were rejected in case of nonexponentialkinetics.

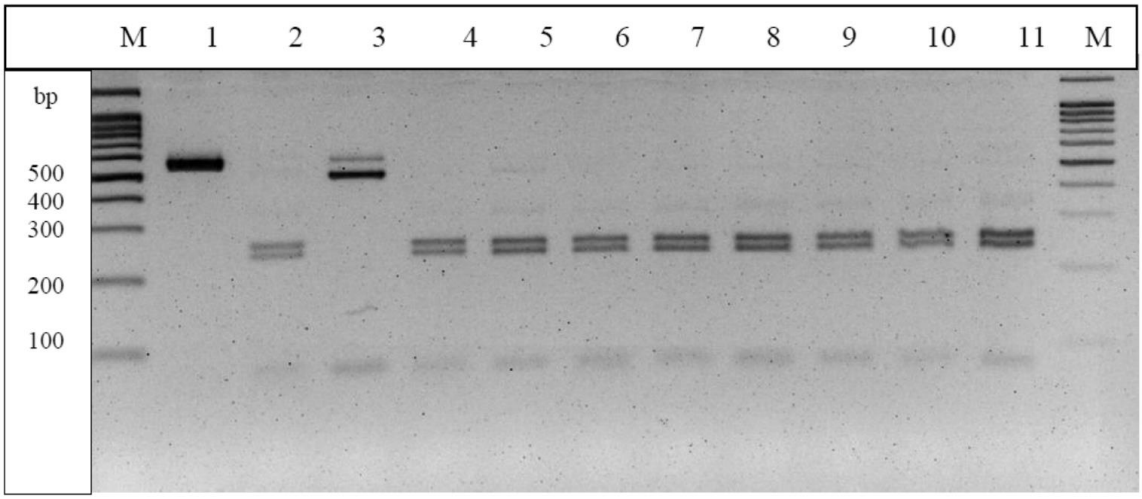
Table 3 Prevalence of *Pfmdr1* point mutation and copy number in *Plasmodium falciparum* from Trat province and Chanthaburi province

Polymorphisms of *Pfmdr1*		**Trat**	**Chantaburi**
Pfmdr1 N86Y	86N	38 (100%, n=38)	12 (80%, n=15)
	86Y	0 (0%, n=38)	3 (20%, n=15)
Pfmdr1 Y184F	184Y	38 (100%, n=38)	24 (100%, n=24)
	184F	0 (0%, n=38)	0 (0%, n=24)
Pfmdr1 S1034C	1034S	32 (100%, n=32)	5 (71.42%, n=7)
	1034C	0 (0%, n=32)	2 (28.57%, n=7)
Copy number of *Pfmdr1*		2.2 ± 0.43	1.48 ± 0.32



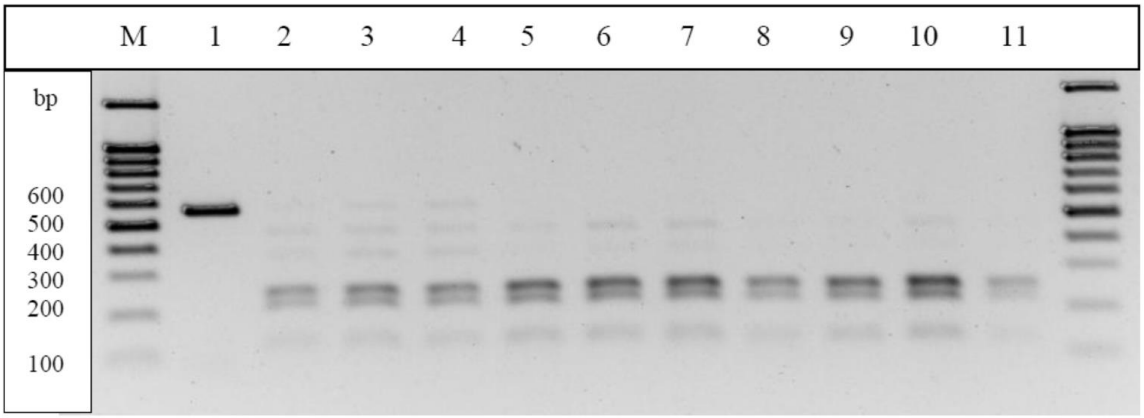
1 = Uncut 2 = 3D7 3 = K1 4 = RC17 5= TD502 6= TD522
7= TD523 8= TD524 9= TD525 10=TD526 11=TD529

Figure 1 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Afl III* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 is mutant-type positive control and 3D7 is wild-type positive control



1 = Uncut 2 = 3D7 3 = K1 4 = BR25 5= KS4 6= TR14
7= BR28 8= BR30 9= BR31 10= BR32 11= BR33

Figure 2 Detection of *Pfmdr1* polymorphism at codon 86, which was digested by *Apo I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 is mutant-type positive control and 3D7 is wild-type positive control



1 = Uncut 2 = 3D7 3 = BR25 4 = BR28 5= BR29 6= BR30
7= BR31 8= BR32 9= BR733 10= CB3 11= CB4

Figure 3 Detection of *Pfmdr1* polymorphism at codon 184, which was digested by *Dra I* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; 3D7 are wild-type positive control

RESULTS

Analysis of *Pfmdr1* gene mutation of *P. falciparum* by PCR-RFLP

73 genomic DNA of *P. falciparum* blood samples (49 samples from Trat and 24 from Chantaburi) were extracted using Chelex-resin according to the method of Plowe and colleague in 1995. The DNA were then analyse for point mutation of *Pfmdr1* gene in the codons 86, 184 and 1034 by PCR-RFLP method. After conducting the PCR with the specific primers of each codon, the amplified products were cut by restriction enzymes and revealed the specific bands size as shown in Table 2. The results of *Pfmdr1* gene point mutation analysed in samples collected from Trat and Chantaburi samples were shown in Table 3. In addition, Figure 1-3 were the example of figures of the PCR products after cutting with restriction enzyme and running on 2 % agarose gel electrophoresis.

The results revealed that there is no mutation in *Pfmdr1* gene in *P. falciparum* from Trat whereas the samples collected from Chantaburi found point mutation in N86Y (20%, n=15), S1034C (28.57%, n=7) and no mutation was found in Y184F.

Analysis of *Pfmdr1* gene copy number in *P. falciparum*

Detection of *Pfmdr1* gene copy number was determined by using TaqMan® real time qPCR. The primers and the probe specific to a conserved region of *Pfmdr1* and the primers and a probe specific to *β-actin* were designed. The *P. falciparum* 3D7 clone was determined in parallel as control samples. 3D7 carry only one gene copy of *Pfmdr1*. At the end of each reaction, cycle threshold (C_T) and melting curves were generated for further analysis. *Pfmdr1* gene was determined by relative quantification between *Pfmdr1* gene and *β-actin* gene based on the $\Delta\Delta C_T$ method. The *β-actin* is housekeeping genes that carry only a single copy of *Pfmdr1* in all parasite isolates and thus allow the comparison of the gene copy number. The target gene (*Pfmdr1*) and reference gene (*β-actin*) were amplified with the same efficiency with an appropriate range of DNA concentrations. The copy number of *Pfmdr1* was calculated using the comparative C_T method, also called $2^{-\Delta\Delta CT}$ method, where $\Delta C_{T,S_{ample}} = C_{T,pfmdr1} - C_{T,reference}$, $\Delta C_{T,S_{ample}}$ is the C_T value for any sample normalized to the endogenous house-keeping gene, and $\Delta\Delta C_T = \Delta C_{T,S_{ample}} - \Delta C_{T,3D7.}$

In all the samples, the $2^{-\Delta\Delta CT}$ refers to N-fold change of *Pfmdr1* copy number relative to 3D7 *Pfmdr1* copy number. Therefore, the 3D7 *Pfmdr1* copy number was calculated by $\Delta C_{T,3D7.} = C_{T,pfmdr1} - C_{T,reference}$ $\Delta\Delta C_T = \Delta C_{T,3D7} - \Delta C_{T,3D7} = 0$. 3D7 *Pfmdr1* copy number = $2^{-\Delta\Delta CT} = 2^{-0} = 2^0 = 1$. For each sample, $\Delta C_{T,E}$ denoting the experimental ΔC_T was determined by $\Delta C_{T,G} - \Delta C_{T,R}$,where $\Delta C_{T,G}$ is the target and $\Delta C_{T,R}$ is the reference C_T. This value was then applied to the comparative $\Delta\Delta C_T$ method: $\Delta\Delta C_T = \Delta C_{T,E} - \Delta C_{T,3D7}$ where the $\Delta C_{T,3D7}$ is the ΔC_T of 3D7. The copy number of *Pfmdr1* in each sample was revealed by $2^{-\Delta\Delta CT}$ as the N-fold copy number of *Pfmdr1* when compare with 3D7 [16].

The results showed that in Trat province exhibited the higher percentage of *P. falciparum* with three or more copies of *Pfmdr1* than in Chantaburi (27.27% and 0%, respectively). The mean *Pfmdr1* copy number in *P. falciparum*

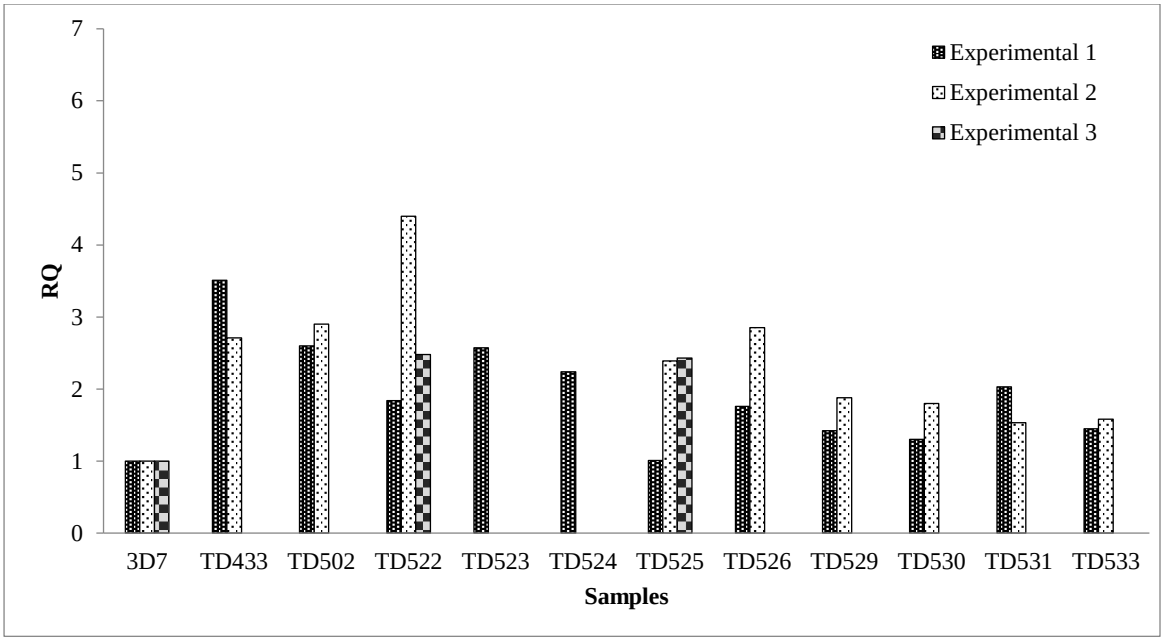


Figure 4 Relative quantification of *Pfmdr1* gene from Trat province.
3D7 is positive control (copy number = 1)

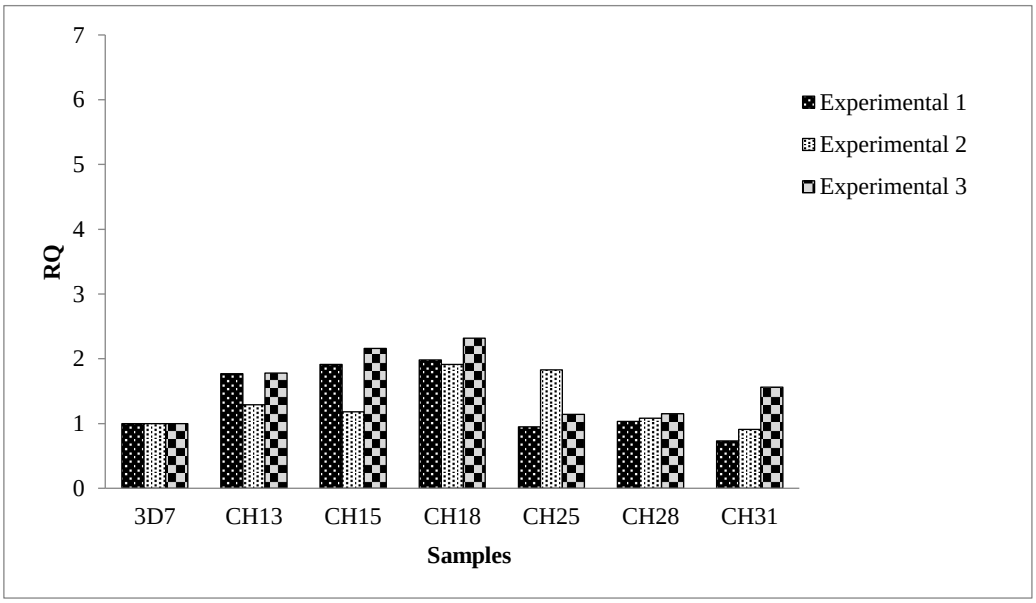


Figure 5 Relative quantification of *Pfmdr1* gene from Chanthaburi province.
3D7 is positive control (copy number = 1)

collected from Trat and Chantaburi were 2.2 and 1.5 respectively, as shown in Table 3. The relative quantification values were shown in Figure 4-5.

DISCUSSION

Multidrug-resistant falciparum malaria is now a serious problem in Southeast Asia, where resistance to chloroquine, mefloquine and quinine are frequently found [17]. This resistance has been restrained to some degree through the use of artemisinin-based combination therapies, which is recommended by the world Health Organization as the first-line treatment of uncomplicated falciparum malaria in endemic areas. In Thailand anti-malarial drugs resistance has been spread over 50 years. Currently, the situation of drugs resistance is more seriously. The evidences both *in vitro* and *in vivo* of *P. falciparum* which is the most resistant strain has been confirmed. There are some studies reported that the falciparum malaria in Thailand already resistant to chloroquine, sulfadoxine-pyrimethamine, mefloquine and quinine [18]. The

declining of mefloquine susceptibility leading to the addition of artesunate in the first-line drugs regimen in Trat, Chantaburi, Sa Kaeo which are located in Thai-Cambodian border, and Tak in Thai-Myanmar border. The resistance of *P. falciparum* is the main factor contributing to the obstruction of malaria control program in Thailand.

The objectives of this study are to determine the distribution of the point mutations of *Pfmdr1* gene and to determine the *Pfmdr1* gene copy number of *P. falciparum* endemic in Thai-Cambodia border. The study areas are 2 provinces, Chantaburi and Trat, which are highly resistant endemic areas. Several studies have shown that single nucleotide polymorphisms and amplification of the *Pfmdr1* gene is associated with *in vitro* response and clinical efficacy of mefloquine, an arylaminoalcohol. Evidence suggests that the *Pfmdr1* gene plays a role in the *in vitro* response to other quinolines such as quinine and lumefantrine and artemisinin derivatives. The results revealed that there was no mutation in *Pfmdr1* gene in *P. falciparum* from Trat where as in Chantaburi found point mutation in N86Y (20%, n=15), S1034C (28.57%, n=7) and no mutation was found in Y184F both in *P. falciparum* collected from Trat and Chantaburi. The prevalence of point mutation at codon 86 and 1034 in *Pfmdr1* gene of *P. falciparum* endemic in Chantaburi province was higher than in Trat province even though they both are situated in Thai-Cambodia border. There are no point mutations at codon 184 in *Pfmdr1* gene of *P. falciparum* endemic both in Chantaburi Province and Trat province. In contrast, *Pfmdr1* copy number in *P. falciparum* endemic in Trat province was higher than in Chantaburi province. The parasites exhibited *Pfmdr1* gene copy number lower than 3 copies were found sensitive to ACTs treatment patients in both of Trat and Chanthaburi provinces. This results was correlated to the previous study that the parasites exhibited Pfmdr1 gene copy number lower than 3 copies were found sensitive to ACTs treatment.

Previously, *P. falciparum* isolates collected in 1988-2003 from this area were genetically characterized. Comparison to the present study, Mungthin et al. [19] identified 86Y 9%, 184F 86%, 1034C18% from Trat and Chanthaburi provinces with a trend of increasing prevalence of wild-type genotypes. The prominent pattern of *Pfmdr1* at condon 86/184/1034 was NFS with prevalence increasing from 40% to 90% and the prevalence of more than one copy number increasing from 17% to 62% during the 10-year period. From the results of this study when compared to the previous study, Mungthin et al., the prevalence of mutant isolates are increasing in some codon which are N86Y and S1034C but in codon Y184F we could not detect any mutation in this codon. In conclusion there is an increasing in mutant isolates in some codons. Their results support to this study that found *P. falciparum* exhibited more than one copy number of *Pfmdr1* 100% in Trat and 50% in Chanthaburi, from 2005-2010 [19]. Moreover, in Thai-Myanmar border area found 86Y 5%, more than one copy number 52% from the samples year 2006-2009 [10].

In addition in southern areas of Thailand, found 86Y 36%, 184F 63%, 1034C 0% in upper southern (Ranong and Chumphon) whereas in lower southern (Yala, Narathiwas, and Songkhla) found 86Y 96%, 184F 3%, 1034C 0.4%. These results show the *Pfmdr1* 184F allele was more common in the parasite from upper southern areas. However, the copy number from upper southern areas was significantly higher than lower southern areas with the mean 2.3 and 1.2 respectively, in year 2009-2010 [20].

In contrast, there are reported in Malaysia that found 86Y 5% from *P. falciparum* collected during 2007-2009 and revealed high predominance of wild type. However, this study found 1246C 5% that is rarely found in Thailand [21].

Pfmdr1 mutation and copy number may be used as an attractive alternative tool to investigate the role of drug resistance of malaria parasites in laboratory studies or large scale epidemiological survey.

CONCLUSION

The *P. falciparum* isolates were observed during the five-year observation period (2006-2010). *Pfmdr1* appear to be the keys genes that modulate multi-drugs resistance in *P. falciparum*. Trat and Chanthaburi provinces are the endemic areas to implications for anti-malarial multi-drugs resistance in Thailand.

The parasites from Trat province exhibited different resistant patterns compared to Chantaburi province even though these two provinces are located close to the border of Thai-Cambodia. The present study showed that *P. falciparum* isolated from different areas along the international border of Thailand exhibited different resistant phenotypic and genotypic patterns. However, this information from this study will be useful for anti-malarial drug policy in Thailand, early detection of emergence of

anti-malarial drugs resistance. New candidate drugs should be adopted at least based on their activity against these phenotypic and genotypic parasites in different areas of Thailand.

ACKNOWLEDGEMENTS

We are grateful to all volunteers for their warm cooperation. We also thank Mrs. Saowanit Vijaikadka for providing blood samples. This work was supported by WHO/SEARO and the Surveillance Center on Health and Public Health Problem, College of Public Health Sciences, Chulalongkorn University. Special thanks to Dr.Tepanata Pompaibool for her helps and valuable suggestions.

REFERENCES

1. World Health Organization [WHO]. International Travel and Health. Geneva: WHO; 2012.
2. Chaijaroenkul W, Wisedpanichkij R, Na-Bangchang K. Monitoring of in vitro susceptibilities and molecular markers of resistance of *Plasmodium falciparum* isolates from Thai-Myanmar border to chloroquine, quinine, mefloquine and artesunate. Acta Trop. 2010 Feb; 113(2): 190-4. doi: 10.1016/j.actatropica.2009.10.016
3. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, et al. Artemisinin resistance in *Plasmodium falciparum* Malaria. N Engl J Med. 2009; 361(5): 455-67. doi: 10.1056/NEJMoa0808859
4. Muhamad P, Chaijaroenkul W, Phompradit P, Rueangweerayut R, Tippawangkosol P, Na-Bangchang K. Polymorphic patterns of *pfcrt* and *pfmdr1* in *Plasmodium falciparum* isolates along the Thai-Myanmar border. Asian Pac J Trop Biomed. 2013 Dec; 3(12): 931-5. doi: 10.1016/s2221-1691(13)60181-1
5. Poyomtip T, Suwandittakul N, Sitthichot N, Khositnithikul R, Tan-ariya P, Mungthin M. Polymorphisms of the *pfmdr1* but not the *pfnhe-1* gene is associated with in vitro quinine sensitivity in Thai isolates of *Plasmodium falciparum*. Malar J. 2012 Jan; 11: 7. doi: 10.1186/1475-2875-11-7
6. Sidhu AB, Valderramos SG, Fidock DA. *Pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. Mol Microbiol. 2005 Aug; 57(4): 913-26. doi: 10.1111/j.1365-2958.2005.04729.x
7. Sidhu AB, Uhlemann AC, Valderramos SG, Valderramos JC, Krishna S, Fidock DA. Decreasing *pfmdr1* copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. J Infect Dis. 2006 Aug; 194(4): 528-35. doi: 10.1086/507115
8. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature. 2000 Feb; 403(6772): 906-9. doi: 10.1038/35002615
9. Duah NO, Matrevi SA, de Souza DK, Binnah DD, Tamakloe MM, Opoku VS, et al. Increased *pfmdr1* gene copy number and the decline in *pfcrt* and *pfmdr1* resistance alleles in Ghanaian *Plasmodium falciparum* isolates after the change of anti-malarial drug treatment policy. Malar J. 2013 Oct; 12(1): 377. doi: 10.1186/1475-2875-12-377
10. Phompradit P, Wisedpanichkij R, Muhamad P, Chaijaroenkul W, Na-Bangchang K. Molecular analysis of *pfatp6* and *pfmdr1* polymorphisms and their association with in vitro sensitivity in *Plasmodium falciparum* isolates from the Thai-Myanmar border. Acta Trop. 2011 Oct-Nov; 120(1-2): 130-5. doi: 10.1016/j.actatropica.2011.07.003
11. Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE. Pyrimethamine and proguanil resistance-conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. Am J Trop Med Hyg. 1995 Jun; 52(6): 565-8.
12. Cowman AF, Karcz S, Galatis D, Culvenor JG. A P-glycoprotein homologue of *Plasmodium falciparum* is localized on the digestive vacuole. J Cell Biol. 1991 Jun; 113(5): 1033-42.
13. Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, Warhurst DC. The tyrosine-86 allele of the pfmdr1 gene of *Plasmodium falciparum* is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. Mol Biochem Parasitol. 2000 Apr; 108(1): 13-23.
14. Duraisingh MT, Cowman AF. Contribution of the *pfmdr1* gene to antimalarial drug-resistance. Acta Trop. 2005 Jun; 94(3): 181-90. doi: 10.1016/j.actatropica.2005.04.008
15. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, et al. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. Lancet. 2004 Jul-Aug; 364(9432): 438-47. doi: 10.1016/s0140-6736(04)16767-6
16. Ferreira ID, Rosario VE, Cravo PV. Real-time quantitative PCR with SYBR Green I detection for estimating copy numbers of nine drug resistance candidate genes in *Plasmodium falciparum*. Malar J. 2006 Jan; 5: 1. doi: 10.1186/1475-2875-5-1
17. Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR. Epidemiology of drug-resistant malaria. Lancet Infect Dis. 2002 Apr; 2(4): 209-18.
18. Wongsrichanalai C, Sirichaisinthop J, Karwacki JJ, Congpuong K, Miller RS, Pang L, et al. Drug resistant malaria on the Thai-Myanmar and Thai-Cambodian borders. Southeast Asian J Trop Med Public Health. 2001 Mar; 32(1): 41-9.
19. Mungthin M, Suwandittakul N, Chaijaroenkul W, Rungsrihirunrat K, Harnyuttanakorn P, Seugorn A, et al. The patterns of mutation and amplification of Plasmodium falciparum pfcrt and pfmdr1 genes in Thailand during the year 1988 to 2003. Parasitol Res. 2010 Aug; 107(3): 539-45. doi: 10.1007/s00436-010-1887-x

20. Mungthin M, Intanakom S, Suwandittakul N, Suida P, Amsakul S, Sitthichot N, et al. Distribution of pfmdr1 polymorphisms in *Plasmodium falciparum* isolated from Southern Thailand. Malar J. 2014 Mar; 13: 117. doi: 10.1186/1475-2875-13-117

21. Atroosh WM, Al-Mekhlafi HM, Mahdy MA, Surin J. The detection of *pfcrt* and *pfmdr1* point mutations as molecular markers of chloroquine drug resistance, Pahang, Malaysia. Malar J. 2012 Aug; 11: 251. doi: 10.1186/1475-2875-11-251