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Cytochrome b mutation and atovaquone susceptibility in *Plasmodium falciparum* isolates from the Thai-Cambodian border during 1990–2010

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ABSTRACT: WHO reported that the efficacy of artemisinin combination-based therapies (ACTs) has decreased around the Thai-Cambodian border. To maintain the efficacy and prolong the life span of ACTs, malarone (an atovaquone and proguanil combination) has been administered in Trat and Chanthaburi Provinces of Thailand since 2009. The mutations of codons 133, 258, 268, 272, 275, 280, 283, and 284 in the *cytochrome b* gene have been reported to be related to malarone resistance. This study investigates the susceptibility in vitro and mutations in the *cytochrome b* gene before and after the administration of malarone. *Plasmodium falciparum* infected blood samples obtained from malaria patients attending malaria clinics in these two provinces were included in this study. Fifteen parasite isolates were subjected to in vitro susceptibility tests against atovaquone. Their mean inhibitory concentrations ranged from 5×10^{-9}–5×10^{-10} M. The DNA sequences from 37 PCR samples revealed no mutations. Therefore the variation of drug susceptibility among these parasites may be unrelated to point mutations in the *cytochrome b* gene.

KEYWORDS: malaria, in vitro drug sensitivity assay

INTRODUCTION

Plasmodium falciparum, a parasite transmitted to humans by *Anopheles* mosquito, causes the most severe malaria. Unlike other malaria parasites, *P. falciparum* has the ability to rapidly transform itself into drug resistant population, especially those spread near the Thai-Cambodian border. This area has been known as an important centre of multidrug resistant development for several decades[1,2]. To control this parasite, development and improvement of several drugs and drug regimens are needed[2]. A few years ago, the World Health Organization recommended the use of artemisinin-based combination therapy (ACTs) as a first-line malaria treatment in endemic areas where high incidence of drug resistance was established. Artemisinin and its derivatives rapidly reduced clinical symptoms and parasite burden. A 3-day artesunate-mefloquine (ARS-MQ) combination is currently being used as the first-line treatment against multidrug resistance malaria in this area. Unfortunately, recent reports from Cambodia showed that some parasites have a slightly reduced in vitro sensitivity to artesunate and a significant prolonged parasite clearance times[3–5]. This suggests that the parasite may have developed resistance against the ARS-MQ combination. In an attempt to delay the development of resistance, new antimalarial drugs are needed for the treatment.

Malarone is a drug of choice and it is a combination of atovaquone and proguanil. Atovaquone has been proposed as an inhibitor of a mitochondrial electron transport process, cytochrome bc1 complex, which collapses the mitochondrial membrane potential[6]. On the other hand, proguanil restrains the function of plasmodial dihydrofolate reductase (DHFR) enzyme and interferes with parasite DNA synthesis[7]. Malarone exhibits high efficacy for the treatment of drug resistant *P. falciparum* near the Thai-Myanmar border[8]. Malarone was first introduced in Trat and Chanthaburi provinces during 2009–2011 in nine areas: Tambon Bo Phloi,

Dan Chumphon, and Nong Bon at Borai District in Trat province; Tambon Ban Laem, Ban Phak Kat, Ban Thap Phai at Pong Nam Ron in Chanthaburi province; Tambon Ban Tha Moon, Ban Thap Chang, Ban Surn Sum at Soi Dao in Chanthaburi province[9]. It must be noted that malarone treatment failures have been reported in other countries and the resistance could be associated with point mutations in the *cytochrome b* (*cyt b*) gene at codons 258[10] and 268[11–19]. Moreover, point mutations at 133, 272, 275, 280, 283, and 284[20] were also reported to be associated with malarone resistance in vitro. The objective of this study was to investigate in vitro susceptibility against atovaquone and the mutations in *cytochrome b* genes before and after the administration of malarone in Thailand. These baseline data are important for monitoring of drug resistance situation in this area.

MATERIALS AND METHODS

Collection of parasites

P. falciparum samples were collected from Trat (TD, BR) and Chanthaburi (CH) provinces at Malaria Clinic during 1990–2011. Forty-one samples were cultured-adapted isolates and have been cryopreserved at Malaria Research Programme, Chulalongkorn University, Thailand. Twenty-two samples were collected using filter papers. The T9/94RC17 and K1CB1 are standard clones used as control clones, their MICs against other antimalarial drugs were previously determined and have been stable for many years.

In vitro drug sensitivity assay

Before the drug assay, the parasites were synchronized to ring stage using D-sorbitol method[21]. Drug susceptibility of malaria isolates against atovaquone was determined by minimum inhibitory concentration assay (MIC)[22,23].

To perform drug susceptibility test, the synchronized parasites were adjusted to 0.3–0.5% parasitaemia with uninfected red blood cells. Then, 10 µl of parasites were added into the well of a 96 micro-well plate which contained 100 µl RPMI complete medium supplemented with antimalarial drugs at various concentrations; 0, 10^{-10}, 5×10^{-10}, 10^{-9}, 5×10^{-9}, and 7.5×10^{-9} M, respectively. Each experiment was performed in triplicate. For comparison, T9/94RC17 and K1CB1 were included in all experiments to standardize the assay condition.

The RPMI medium (with or without drug/s) was changed daily. After 72 h, thin blood films stained with Giemsa were observed under light microscope. The minimum drug concentration at which the survived parasite cannot be detected was reported as the minimum inhibitory concentration (MIC) of the tested isolates or clones.

Genomic DNA extraction

The genomic DNA of *P. falciparum* was extracted by phenol-chloroform method as described by Snounou[24]. The DNA stock solutions were stored in a −20 °C freezer throughout the experiment.

Polymerase Chain reaction

The *cyt b* gene was amplified by nested-PCR method[25]. One microlitre of diluted DNA template was added into the PCR reaction. The concentration of PCR reagents in the mixture were adjusted as followed: 1× *Taq* DNA polymerase buffer, 2 mM $MgCl_2$, 0.25 mM dNTP, 0.25 mM of forward and reverse primers, and 2.5 unit *Taq* DNA polymerase in a 20 µl volume reaction. The temperature cycle was set as denaturation (95 °C, 30 s), annealing (50 °C, 30 s), and extension (72 °C, 1 min) for 30 cycles using the *Veriti* 96-Well Thermal Cycler from Applied Biosystems. The forward and reverse primers were Plasmodium F (5′-GCCTAGACGTATTCCTGATTATCC AG-3′), Plasmodium R (5′-CTCCCTATCATGTCTTGC TAACGGC-3′), falciparum specific F (5′-GATGGAATA TGATTTGTTCTATTGGG-3′), and falciparum specific R (5′-CCTTACGGTCTGATTTGTTCCGCTC-3′).

The amplified products were determined by agarose gel electrophoresis[26]. Graphical information was captured by the Autochemi system (UVP, LLC).

DNA sequencing

The *cyt b* PCR products were purified and sequenced by Bio Basic Canada Inc. Sequences from both strands were aligned with BIOEDIT. The sequences of each isolates were deduced from the matched sequences in both strands. Finally, all sequences were compared and analysed for gene mutation.

RESULTS

From 41 *P. falciparum* samples, only 15 isolates were successfully revived. Most of the cultured isolates either became contaminated or died. Fourteen isolates were collected from Trat province during 1990–2008, before malarone was introduced as the first-line treatment. An isolate, TD559, was collected in 2010, one year after malarone was used. On the other hand, among the 22 samples collected by filter paper, 5 samples were collected from Trat

Table 1 Time of collection and types of *Plasmodium falciparum* samples.

Years	Atovaquone usage	Types of samples	
		in vitro culture	filter paper
1990–2008	No	TD3, TD498, TD500, TD506, TD530, TD533, TD541, TD542, TD544, TD545, TD547, TD550, TD554, TD556	BR17, BR26, BR102, TD373, TD371
2009–2011	Yes	TD559	CH1, CH2, CH3, CH4, CH6, CH7, CH8, CH9, TD560, TD68, TD73, TD76, TD77, TD79,TD80, TD81,TD82

T9/94RC17 and K1CB1 were included as control clones.

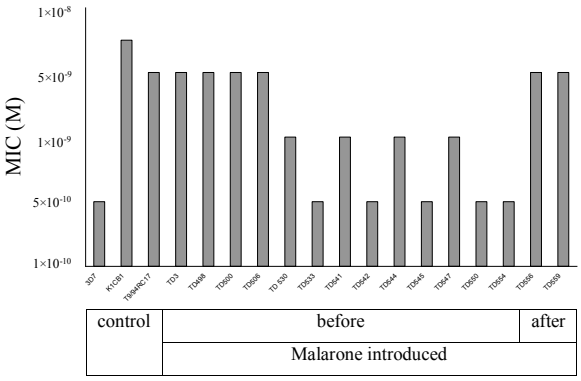

Fig. 1 The MIC of *P. falciparum* samples tested against atovaquone. 3D7, K1CB1, and T9/94RC17 were included as control clones. The parasite samples can be categorized as before and after malarone was introduced.

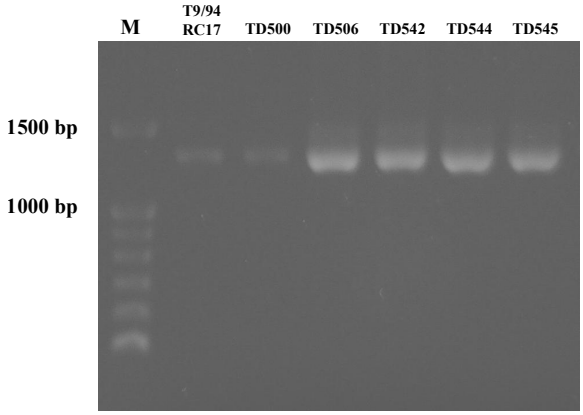

Fig. 2 PCR products of the *cyt b* gene from different isolates of *P. falciparum* separated by agarose gel electrophoresis. M: DNA markers (1 kb Plus 1500 pb); lane 1: PCR product of T9/94RC17; lanes 2–6: PCR products of *P. falciparum* isolates.

province during 1990–2008 while 17 samples were received from Trat and Chanthaburi provinces during 2009–2011 (Table 1).

All fifteen parasite isolates, from Trat province, showed different levels of MIC. These isolates could be divided into three groups according to their MIC values, i.e., 5×10^{-10} M (TD533, TD542, TD545, TD550, and TD554), 10^{-9} M (TD530, TD541, TD544, and TD547), and 5×10^{-9} M (TD3, TD498, TD500, TD506, TD556, and TD559) (Fig. 1). 3D7 and K1CB1 clones gave the lowest (5×10^{-10} M) and highest MIC (7.5×10^{-9} M), respectively. In comparison, T9/94RC17 clone showed moderate MIC (5×10^{-9} M) in this experiment.

The PCR products of *cyt b* gene were detected as a 1254 bp fragment compared to the expected size at 1131 bp (Fig. 2). No size polymorphism could be detected from the isolates and the control clones by agarose gel electrophoresis.

All PCR products were sequenced and analysed. The shortest readable sequences of the PCR products were from the 360th to the 902th which covered all reported mutations in the *cyt b* gene. There was no mutation detected in the readable sequences, especially at $133^{M/I}$, $258^{I/M}$, $268^{Y/S}$, $272^{K/R}$, $275^{P/T}$, $280^{G/D}$, $283^{L/I}$, $284^{V/K}$ positions (amino acid residue of the wild type/mutated amino acid)

DISCUSSION

Two groups of *P. falciparum* samples had been analysed, those collected before and after the malarone had been introduced. These parasites showed different levels of MIC against atovaquone, even though atovaquone had never been used in Thailand. From the in vitro drug susceptibility assay against atovaquone, 3D7, T9/94RC17, K1CB1 exhibited variable MIC levels (Fig. 1). The K1 isolate was originally collected from Kanchanaburi province in 1979 and has been continuously maintained in the laboratory. The K1CB1 was cloned

from K1 isolate in 1991. None of the samples from Trat and Chanthaburi showed the same MIC level as the K1CB1 clone. It must be noted however that the MIC levels of TD530 through TD554, collected during 2005–2008, were lower than the MIC collected before (1990–2003) and after (2009) this period.

The presence of point mutations of *cytochrome b* gene at the codon 258[10], 268[11–19] and codon 133, 272, 275, 280, 283, and 284[20] have been proposed to be related to atovaquone resistance. In this experiment, the *cyt b* gene from 37 samples exhibited the same nucleotide sequences in all readable sequences in these positions. Similarly, no mutation at the codon 268 was detected among *P. falciparum* samples collected from Thai-Myanmar or Thai-Cambodian border[27,28]. This suggested that the MIC value of the samples used in this experiment may not be related to previously reported mutations.

If previously proposed mutations are related to atovaquone resistance, these results suggest that atovaquone should still be effective against falciparum parasite in Thai-Cambodian border areas. Although none of tested isolates were categorized as high MIC against atovaquone similar to the K1CB1 clone, the misuse of atovaquone may help select these parasites and, finally, increase the tendency towards atovaquone resistance in the future.

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REFERENCES

1. Vijaykadga S, Rojanawatsirivej C, Cholpol S, Phoungmanee D, Nakavej A, Wongsrichanalai C (2006) In vivo sensitivity monitoring of mefloquine monotherapy and artesunate-mefloquine combinations for the treatment of uncomplicated falciparum malaria in Thailand in 2003. *Trop Med Int Health* **11**, 211–9.
2. Lopes D, Rungsihirunrat K, Nogueira F, Seugorn A, Gil JP, do Rosário VE, Cravo P (2002) Molecular characterization of drug-resistant *Plasmodium falciparum* from Thailand. *Malaria J* **1**, 12.
3. Thimasarn K, Sirichaisinthop J, Chanyakhun P, Palananth C, Rooney W (1997) A comparative study of artesunate and artemether in combination with mefloquine in multidrug resistance falciparum malaria in eastern Thailand. *Southeast Asian J Trop Med Publ Health* **28**, 465–71.
4. Wongsrichanalai C, Sirichaisinthop J, Karwacki JJ, Congpuong K, Miller RS, Pang L, Thimasarn K (2001) Drug resistant malaria on the Thai-Myanmar and Thai-Cambodian borders. *Southeast Asian J Trop Med Publ Health* **32**, 41–9.
5. Bunnag D, Kanda T, Karbwang J, Thimasarn K, Pungpak S, Harinasuta T (1995) Artemether-mefloquine combination in multidrug resistant falciparum malaria. *Trans Roy Soc Trop Med Hyg* **89**, 213–5.
6. Srivastava IK, Rottenberg H, Vaidya AB (1997) Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite. *J Biol Chem* **272**, 3961–6.
7. Reynolds MG, Roos DS (1998) A biochemical and genetic model for parasite resistance to antifolates *Toxoplasma gondii* provides insights into pyrimethamine and cycloguanil resistance in *Plasmodium falciparum*. *J Biol Chem* **273**, 3461–9.
8. Krudsood S, Patel SN, Tangpukdee N, Thanachartwet W, Leowattana W, Pornpininworakij K, Boggild AK, Looareesuwan S, Kain KC (2007) Efficacy of atovaquone-proguanil for treatment of acute multidrug-resistant *Plasmodium falciparum* malaria in Thailand. *Am J Trop Med Hyg* **76**, 655–8.
9. BVBD (2009) *Malaria Control Programme in Thailand*, Bureau of the Vector-borne Diseases, Department of Disease Control, Ministry of Public Health.
10. Plucinski MM, Huber CS, Akinyi S, Dalton W, Eschete M, Grady K, Silva-Flannery L, Mathison BA, et al (2014) Novel mutation in cytochrome b of *Plasmodium falciparum* in one of two atovaquone-proguanil treatment failures in travelers returning from same site in Nigeria. *Open Forum Infect Dis* **1**, doi: 10.1093/ofid/ofu059.
11. Fivelman QL, Butcher GA, Adagu IS, Warhurst DC, Pasvol G (2002) Malarone treatment failure and in vitro confirmation of resistance of *Plasmodium falciparum* isolate from Lagos, Nigeria. *Malaria J* **1**, 1.
12. Schwöbel B, Alifrangis M, Salanti A, Jelinek T (2003) Different mutation patterns of atovaquone resistance to *Plasmodium falciparum* in vitro and in vivo: rapid detection of codon 268 polymorphisms in the cytochrome b as potential in vivo resistance marker. *Malaria J* **2**, 5.
13. David KP, Alifrangis M, Salanti A, Vestergaard LS, Rønn A, Bygbjerg I (2003) Atovaquone/proguanil resistance in Africa: a case report. *Scand J Infect Dis* **35**, 897–8.
14. Farnert A, Lindberg J, Gil JP, Swedberg G, Berqvist Y, Thapar MM, Lindegardh N, Berezcky S, et al (2003) Evidence of *Plasmodium falciparum* malaria resistant to atovaquone and proguanil hydrochloride: case reports. *Br Med J* **326**, 628–9.
15. Schwartz E, Bujanover S, Kain KC (2003) Genetic confirmation of atovaquone-proguanil–resis-

tant *Plasmodium falciparum* malaria acquired by a nonimmune traveler to East Africa. *Clin Infect Dis* **37**, 450–1.
16. Wichmann O, Muehlberger N, Jelinek T, Alifrangis M, Peyerl-Hoffmann G, Mühlen M, Grobusch MP, Gascon J, et al (2004) Screening for mutations related to atovaquone/proguanil resistance in treatment failures and other imported isolates of *Plasmodium falciparum* in Europe. *J Infect Dis* **190**, 1541–6.
17. Kuhn S, Gill MJ, Kain KC (2005) Emergence of atovaquone-proguanil resistance during treatment of *Plasmodium falciparum* malaria acquired by a nonimmune North American traveller to West Africa. *Am J Trop Med Hyg* **72**, 407–9.
18. Musset L, Pradines B, Parzy D, Durand R, Bigot P, Le Bras J (2006) Apparent absence of atovaquone/proguanil resistance in 477 *Plasmodium falciparum* isolates from untreated French travellers. *J Antimicrob Chemother* **57**, 110–5.
19. Musset L, Bouchaud O, Matheron S, Massias L, Le Bras J (2006) Clinical atovaquone-proguanil resistance of *Plasmodium falciparum* associated with cytochrome b codon 268 mutations. *Microb Infect* **8**, 2599–604.
20. Korsinczky M, Chen N, Kotecka B, Saul A, Rieckmann K, Cheng Q (2000) Mutations in *Plasmodium falciparum* cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site. *Antimicrob Agents Chemother* **44**, 2100–8.
21. Lambros C, Vanderberg JP (1979) Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol* **65**, 418–20.
22. Thaithong S, Beale GH, Chutmongkonkul M (1983) Susceptibility of *Plasmodium falciparum* to five drugs: an in vitro study of isolates mainly from Thailand. *Trans Roy Soc Trop Med Hyg* **77**, 228–31.
23. Seugorn A, Siripoon N, Kanchanakarn N, Rungsihirunrat K, Pumpaibool T, Vichaikatka S, Thaithong S, Harnyuttanakorn P (2007) Drug susceptibility of *Plasmodium falciparum* collected from different areas of Thailand during 2000–2001. *J Health Res* **21**, 119–24.
24. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN (1993) Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infection. *Mol Biochem Parasitol* **58**, 283–92.
25. Mullis KB (1990) The unusual origin of the polymerase chain reaction. *Sci Am* **262.4**, 56–61, 64–5.
26. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
27. Naoshima-Ishibashi Y, Iwagami M, Kawazu S, Looareesuwan S, Kano S (2007) Analyses of cytochrome b mutations in *Plasmodium falciparum* isolates in Thai-Myanmar border. *Trav Med Infect Dis* **5**, 132–4.
28. Khositnithikul R, Tan-ariya P, Mungthin M (2008) In vitro atovaquone/proguanil susceptibility and characterization of the *cytochrome b* gene of *Plasmodium falciparum* from different endemic regions of Thailand. *Malaria J* **7**, 23.