

## Molecular Detection of Mutations in the Propeller Domain of Kelch 13 and *pfmdr1* Copy Number Variation in *Plasmodium falciparum* Isolates from Thailand Collected from 2002 to 2007

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**Abstract.** We determined the prevalence of Kelch 13 mutations and *pfmdr1* copy number in samples collected from the Thailand–Myanmar border, the Thailand–Cambodia border, and southern Thailand from 2002 to 2007. C580Y was the most prevalent in Trat (Thailand–Cambodia border) and Ranong (Thailand–Myanmar border) at 42% (24/57) and 13% (6/48), respectively. Less predominant mutations were also identified including R539T (7%, 4/57) and Y493H (2%, 1/57) in Trat, P574L (6%, 3/48) and P553L (2%, 1/48) in Ranong, and N537I and D452E (7%, 1/15) in Sangkhlaburi (Thailand–Myanmar border). Samples from Mae sot (33%, 11/33) harbored the highest percentage of multiple *pfmdr1* copies, followed by Trat (18%, 10/57), Chiang Dao in 2003 (13%, 4/30), Phang Nga (5%, 2/44), and Chiang Dao in 2002 (4%, 1/26). This retrospective study provides geographic diversity of K13 and *pfmdr1* copies and the emergence of these molecular markers in Thailand, an important background information for future surveillance in the region.

Artemisinin-based combination therapy (ACT) has been used as the first line of treatment of an uncomplicated *Plasmodium falciparum* infection in Thailand since 1995.<sup>1</sup> Extensive use of ACTs in the Greater Mekong Subregion (GMS) has raised concerns of independent emergence or spread of ACT-resistant *P. falciparum*. Clinical treatment failure attributed to artemisinin (ART) resistance in *P. falciparum* was first confirmed in Cambodia along the Thailand–Cambodia border in 2007.<sup>2,3</sup>

Validated molecular markers have been widely used as identification and prediction tools for the emergence of drug-resistant *P. falciparum* in new locations. Clinical ART resistance is defined as a delayed parasite clearance, a parasite clearance half-life (PC<sub>1/2</sub>), which is greater than 5 hours.<sup>4</sup> Mutations in the Kelch 13 (K13) propeller domain were shown to be strongly associated with delayed parasite clearance making it a good molecular marker for predicting clinical ART resistance. Nonsynonymous K13 mutations segregate into two distinct geographical areas where C580Y, R539T, Y493H, and I543T are commonly found in Cambodia, Vietnam, and Laos, respectively, whereas F446I, N458Y, P574L, and R561H are more specific to the western part of Thailand, Myanmar, and China, respectively.<sup>5</sup> An increase in *pfmdr1* copy number has been linked to *P. falciparum* diminished susceptibility to mefloquine.<sup>6</sup> Therefore, the surveillance of these molecular markers is useful to track the rise and spread of anti-malarial drug resistance in *P. falciparum*.

This study was approved by the Thai Ministry of Public Health (MoPH, Ref. no. 60/2559). Participants from studies in which the samples were collected from provided written informed consents for future use of their blood. This study aimed to determine the presence and track the emergence of K13 and *Pfmdr1* copy number over two decades. A total of 253 *P. falciparum* infected blood samples collected from six provinces in Thailand from 2002 to 2007 were assessed for a prevalence of K13 mutations and *pfmdr1* copy number. Of these

samples, 152 were collected along the Thailand–Myanmar border (56 samples from Chiang Dao in 2002 and 2003, 33 samples from Mae Sot in 2004, 48 samples from Ranong in 2004, and 15 samples from Sangkhlaburi in 2007), whereas in 2005, 57 and 44 samples were collected from Trat, along the Thailand–Cambodia border, and Phang nga in southern Thailand, respectively. Genomic DNA was extracted from ethylenediaminetetraacetic acid (EDTA) whole blood using EZ1 DNA blood kit with automated EZ1 Advanced XL purification system (QIAGEN, Valencia, CA). *Plasmodium* speciation was confirmed by using multiplex real-time polymerase chain reaction (PCR) assay.<sup>7</sup> Mutations in the propeller domain of *P. falciparum* K13 were determined by nested PCR and DNA sequencing.<sup>4</sup> *Pfmdr1* copy number was determined by TaqMan real-time PCR methods by using  $\beta$ -tubulin as a house-keeping gene and calculated using  $2^{-\Delta\Delta Ct}$  method.<sup>6</sup>

K13 mutations were successfully determined in 251/253 samples (99.2%). Geographic distribution of K13 propeller domain polymorphisms is shown in Figure 1. No K13 mutations were found in samples from Chiang Dao and Mae Sot. The highest proportion of ART-resistance confirmed K13 mutations were found in Trat, with C580Y as the most common mutation (42%, 24/57), followed by R539T (7%, 4/57) and Y493H (2%, 1/57). C580Y was also detected in Ranong and Phang Nga, which are geographically distant from the Thailand–Cambodia border, at 13% (6/48) and 2% (1/44), respectively. The K13 mutations R539T and Y493H were only found in Trat, whereas P574L and P553L, ART-resistance associated K13 mutations were found in Ranong at 6% (3/48) and 2% (1/48), respectively. Although the sample size for Sangkhlaburi was small, N537I and D452E were observed (each at 7%, 1/15).

The *pfmdr1* copy number was successfully determined in 208/253 samples (82.2%). The highest proportion of samples harboring multiple *pfmdr1* copies was found in samples from Mae Sot (33%, 11/33), followed by Trat (18%, 10/57), Chiang Dao in 2003 (13%, 4/30), Phang Nga (5%, 2/44), and Chiang Dao in 2002 (4%, 1/26). All samples from Ranong and Sangkhlaburi harbored a single *pfmdr1* copy (Table 1).

The information of K13 propeller domain polymorphisms together with *pfmdr1* copy number for 208 samples (82.2%) are shown in Figure 2. An increase in samples harboring

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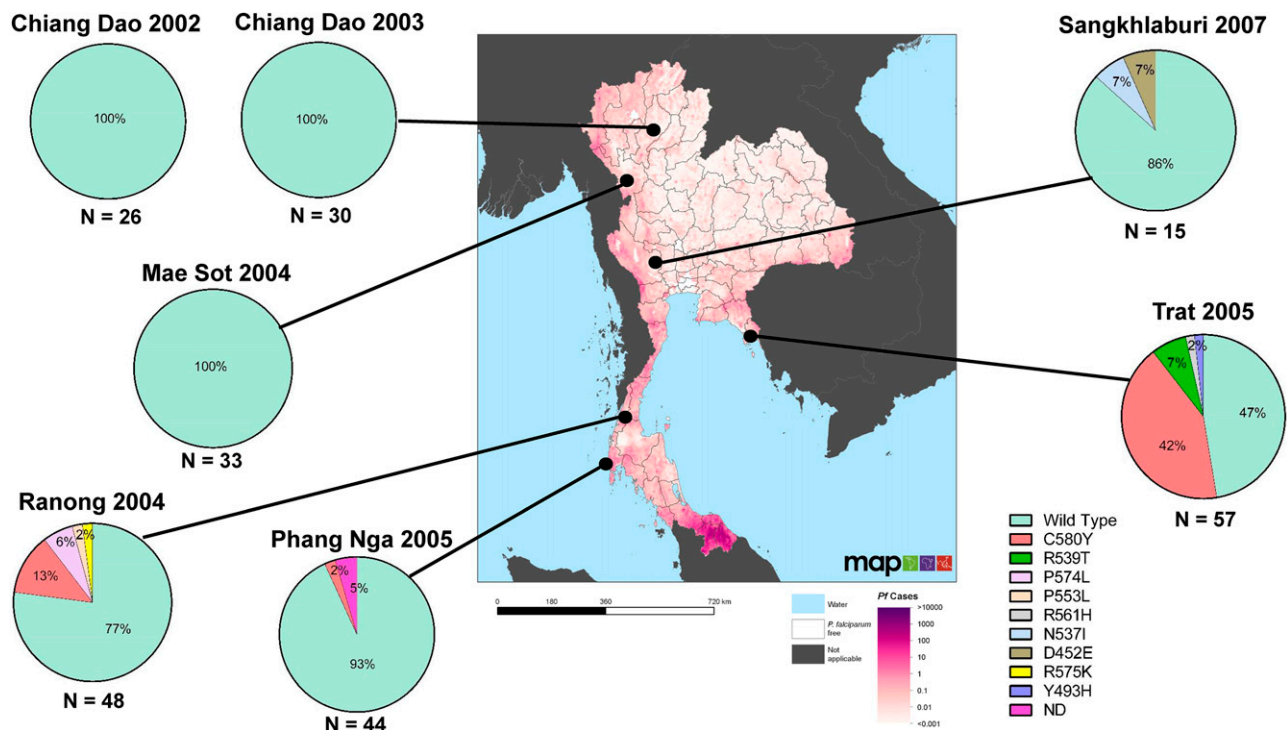


FIGURE 1. Geographic distribution of *Plasmodium falciparum* K13 mutations collected in six provinces in Thailand from 2002 to 2007. The results are depicted on the spatial distribution of *P. falciparum* cases in Thailand in 2007 (Malaria Atlas Project). This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

multiple *pfmdr1* copies with wild-type K13 was observed from 2002 to 2004 in Chiang Dao 2002, Chiang Dao 2003, and Mae Sot 2004. Nonsynonymous K13 mutations were first detected in 2004 and in 2005 and 2007, all with a single *pfmdr1* copy except in Trat in 2005 with K13 mutations combined with either single or multiple copy of *pfmdr1*. Interestingly, multiple *pfmdr1* copies with mutated K13 was observed only in samples from Trat.

Other studies have reported mutations and amplifications of candidate markers conferring antimalarial drug resistance along borders of Thailand with Myanmar, and Cambodia, and in southern Thailand.<sup>8,9</sup> The earliest report of mutations in K13 in Thailand was in Trat in 1991, but those mutations were not associated with ART resistance as classified by the WHO.<sup>10</sup> This study provides retrospective information on prevalence and distribution of K13 mutations in Thailand as early as 2002, and to date, the C580Y mutation found in Ranong is the earliest observation of C580Y mutation in Thailand. This finding is 3 years before other retrospective

analyses of K13 prevalence in Thai *P. falciparum* isolates,<sup>11,12</sup> although 3 years after K13 was found in neighboring Cambodia. R539T, another confirmed mutation associated with ART, was also observed in Trat. Both C580Y and R539T have been reported to be the most prevalent in Cambodia, and may have emerged in Trat via border-crossing movements; therefore, it is possible that K13 mutations were present in Trat before 2005 and present in Thailand before 2004.

A recent report by Khammanee et al. 2019 also observed C580Y in samples from Ranong between 2012 and 2014 and from Phang nga in 2015, suggesting the persistence of C580Y in this area.<sup>8</sup> The proximity of Ranong to Myanmar may also explain our observation of P574L, which is more prevalent in Myanmar. Moreover, parasites carrying C580Y allele were previously shown, via K13 microsatellites, to be distinct between Thailand–Myanmar and Thailand–Cambodia border

TABLE 1  
*Pfmdr1* copy number of *Plasmodium falciparum* samples from six provinces in Thailand collected from 2002 to 2007

Sample location	Year	N	<i>Pfmdr1</i> copy number		
			Single (%)	Multiple (%)	Not determined (%)
Chiang Dao	2002	26	17 (65)	1 (4)	8 (31)
Chiang Dao	2003	30	26 (87)	4 (13)	0 (0)
Mae Sot	2004	33	19 (58)	11 (33)	3 (9)
Ranong	2004	48	41 (85)	0 (0)	7 (15)
Phang Nga	2005	44	29 (66)	2 (5)	13 (29)
Trat	2005	57	33 (58)	10 (18)	14 (24)
Sangkhlaburi	2007	15	15 (100)	0 (0)	0 (0)

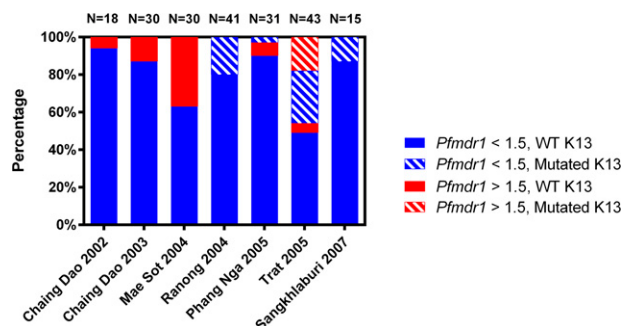


FIGURE 2. *Pfmdr1* copy number and K13 mutations in *Plasmodium falciparum* samples from six provinces in Thailand from 2002 to 2007. This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

areas, suggesting differences in origin and migration along these borders.<sup>9</sup> Therefore, assessing genetic relatedness among the C580Y alleles found in Ranong, Phang Nga, and Trat would be of interest to determine the spread of these parasites across different regions of Thailand.

Multiple *pfmdr1* copies has been shown to be associated with a decrease in vitro sensitivity to mefloquine<sup>6</sup> and it was associated with mefloquine-treatment failures in Cambodia, in which *P. falciparum* recurrent isolates had a higher *pfmdr1* copies than *P. falciparum* isolates taken at the start of treatment.<sup>13</sup> Our study showed that the highest prevalence of multiple *pfmdr1* copies was found in Mae Sot, which could reflect long-term use of mefloquine monotherapy since the mid-1980s. In 2016, Thailand changed malaria treatment policy from artesunate-mefloquine (AS-MQ) to dihydroartemisinin-piperaquine (DHA-PIP) countrywide and the prevalence of multiple *pfmdr1* copies has subsequently been shown to be declining in both eastern and western borders of Thailand.<sup>11</sup> However, *pfmdr1* copy number should be continuously monitored as the reduction of AS-MQ efficacy results from a combination of K13 mutations and multiple *pfmdr1* copies.<sup>14</sup> Cambodia changed the first-line treatment to DHA-PIP in 2012, but because of rapid loss of efficacy, switched back to AS-MQ. A similar situation was unfolding in the neighboring Thailand, and high percentages of failures to DHA-PIP were reported in Sisaket and Ubon Ratchathani provinces.<sup>15</sup> For these regions, in 2019 the MoPH selected an alternative ACT, artesunate-pyronaridine (Pyramax), to be used, whereas DHA-PIP has remained as the treatment of the rest of the country.<sup>16</sup> An efficacy of Pyramax is currently under evaluation in clinical trials by AFRIMS, clinicaltrials.gov identifier: NCT03726593.

Molecular evidence of ACT compromise is evident from isolates carrying both K13 mutations and multiple *pfmdr1* copies, collected in Trat Province over 15 years ago. Unfortunately, a correlation between genotypic and phenotypic data cannot be made because of the lack of in vitro susceptibility testing of these samples. Even as national policy was shifted away from mefloquine, resistance began to mount to other partner drugs and molecular markers to drugs such as piperaquine are now in use for monitoring. If mutations become additive, such as was suggested by emergence of triple mutant parasites (K13, *pfmdr1*, and *plasmepsin 2* copy number) in Cambodia,<sup>17</sup> a need for alternative regimens are warranted. However, ART parasites without K13 mutations have been demonstrated, suggesting that mutations in other genes may be involved.<sup>18</sup> Mutations in *pfcoronin* and *pfatg18* have demonstrated a reduction in ART susceptibility in non-K13-mutant parasites<sup>19,20</sup>; therefore, expanded molecular surveillance is essential for tracking resistance parasites.

In summary, this study provided retrospective information on K13 mutations and *pfmdr1* copy number variation in *P. falciparum* isolates from different areas in Thailand dating back to 2002. Data presented confirm K13-resistance mutations arose in Thailand as early as 2004, before the recognition of the first verified ART resistance in Thailand–Cambodia border 3 years later. The subsequent increase in proportion of isolates with K13 mutations and multiple *pfmdr1* copies warrants continued investments in alternative therapies and continuous surveillance of resistance molecular markers with accompanying in vitro susceptibility information.

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