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# Molecular Markers and *In Vitro* Susceptibility to Doxycycline in *Plasmodium falciparum* Isolates from Thailand

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**Determinations of doxycycline 50% inhibitory concentrations ($IC_{50}$) for 620 isolates from northwest Thailand were performed via the isotopic method, and the data were analyzed by the Bayesian method and distributed into two populations (mean $IC_{50}$s of 13.15 μM and 31.60 μM). There was no significant difference between the group with low $IC_{50}$s versus the group with high $IC_{50}$s with regard to copy numbers of the *Plasmodium falciparum tetQ* (*pftetQ*) gene ($P = 0.11$) or *pfmdt* gene ($P = 0.87$) or the number of PfTetQ KYNNNN repeats ($P = 0.72$).**

The World Health Organization (WHO) recommends doxycycline in combination with quinine or artesunate as the second-line treatment for uncomplicated *Plasmodium falciparum* malaria (1). Doxycycline is currently one of the recommended chemoprophylactic regimens for travelers visiting areas of malaria endemicity, particularly in countries with multiple-drug resistance. The prophylactic failure of doxycycline against *P. falciparum* may be explained by drug resistance, but this has not yet been documented. Indeed, -cycline resistance in *Plasmodium* has been documented only as a consequence of drug pressure in a *P. berghei* murine malaria model (2).

Recent studies have suggested that *P. falciparum mdt* (*pfmdt*) and *pftetQ* copy numbers are potential molecular markers of decreased *in vitro* susceptibility to doxycycline in African *P. falciparum* isolates (3, 4). In addition, isolates with PfTetQ KYNNNN motif repeats have been associated with reduced *in vitro* susceptibility to doxycycline and with a significantly greater probability of a 50% inhibitory concentration ($IC_{50}$) greater than the doxycycline resistance threshold of 35 μM (3, 5).

The objective of this study was to evaluate for the first time the distribution of doxycycline $IC_{50}$s for *P. falciparum* isolates collected in Asian patients and to validate the use of the *pftetQ* and *pfmdt* genes as molecular markers of decreased *in vitro* susceptibility to doxycycline.

Clinical isolates were obtained from patients with acute *P. falciparum* malaria attending Shoklo Malaria Research Unit (SMRU) clinics between 2001 and 2010. The SMRU clinics are all located along the Thai-Myanmar border. Isolates were collected from primary infections with a parasite density of at least 5 parasites/1,000 red blood cells. Samples were kept at room temperature before being transported to the main laboratory, where they were immediately tested *in vitro*. The fresh parasite isolate samples were obtained as part of prospective clinical evaluations of antimalarial drug therapy. Written informed consent translated into the patient's own language was obtained from each participant, whose consent signature was witnessed. The studies were approved by the Ethics Committees of the Faculty of Tropical Medicine, Mahidol University, and Oxford University. All cases were microscopically confirmed to be *falciparum* malaria.

*In vitro* drug susceptibility was determined by the hypoxanthine uptake inhibition assay, which has been described previously (6). The reproducibility of the $IC_{50}$ measurements was assessed regularly using cloned *P. falciparum* strain K1 (Table 1). There was a significant reduction in the doxycycline median $IC_{50}$ for strain K1 in 2003 ($P < 0.0001$). The doxycycline $IC_{50}$s for the 620 isolates ranged from 0.21 to 55.44 μM, with a mean of 14.0 μM ± 6.5 μM. The average parameter estimates for the $IC_{50}$s and their distribution by year are given in Table 1 and in Fig. 1. There were significant differences in the doxycycline median $IC_{50}$s for the sample isolates collected during the study period of 2001 to 2010. The reduction in the doxycycline median $IC_{50}$ in sample isolates collected in 2003 can be explained only by a bias in methodology. Considering the reduction in the doxycycline median $IC_{50}$ for strain K1 in 2003, only 7 isolates of 620 (1.1%) had a doxycycline $IC_{50}$ greater than 35 μM, which was the threshold determined for reduced susceptibility to doxycycline (7), demonstrating that isolates with reduced susceptibility to doxycycline (according to the $IC_{50}$ values) were rare even in Thailand, a geographic area known for multiple drug resistance. This cutoff value of 35 μM was determined for an exposure to doxycycline from 42 h to 48 h (7). A cutoff for *in vitro* resistance is defined for a specific methodology. For example, the *in*

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**TABLE 1** Statistical analysis of the 620 *P. falciparum* Thai isolates and K1 strain *in vitro* responses ($IC_{50}$ in μM) to doxycycline by year

| Parameter | Value | | | | | | |
|---|---|---|---|---|---|---|---|
| | 2001 | 2002 | 2003 | 2007 | 2008 | 2009 | 2010 |
| *Plasmodium falciparum* clinical isolates | | | | | | | |
| No. of isolates | 173 | 244 | 91 | 31 | 24 | 45 | 12 |
| Minimum $IC_{50}$ | 2.37 | 0.83 | 0.21 | 7.89 | 9.04 | 7.08 | 14.42 |
| 25% percentile | 11.99 | 9.66 | 5.13 | 12.05 | 11.66 | 15.91 | 17.41 |
| Median | 14.56 | 12.45 | 8.93 | 14.77 | 14.56 | 17.79 | 19.86 |
| 75% percentile | 18.44 | 16.01 | 12.71 | 17.82 | 19.13 | 20.16 | 21.17 |
| Maximum $IC_{50}$ | 29.28 | 55.44 | 30.48 | 21.5 | 27.33 | 33.36 | 29.26 |
| Mean | 15.16 | 13.44 | 9.61 | 15.08 | 15.89 | 18.37 | 19.93 |
| SD | 5.20 | 7.27 | 6.04 | 3.60 | 5.10 | 5.05 | 3.67 |
| SE | 0.40 | 0.47 | 0.63 | 0.64 | 1.042 | 0.75 | 1.06 |
| Lower 95% CI of mean | 14.38 | 12.52 | 8.36 | 13.76 | 13.73 | 16.86 | 17.6 |
| Upper 95% CI of mean | 15.94 | 14.36 | 10.87 | 16.4 | 19.89 | 16.86 | 22.26 |
| *Plasmodium falciparum* K1 clone | | | | | | | |
| No. of isolates | 6 | 6 | 7 | 4 | 4 | 10 | 6 |
| 25% percentile | 15.82 | 13.81 | 8.59 | 11.79 | 8.54 | 15.89 | 17.52 |
| Median | 17.47 | 14.37 | 8.9 | 12.04 | 11.33 | 18.60 | 18.60 |
| 75% percentile | 18.85 | 15.06 | 9.54 | 12.28 | 13.76 | 21.93 | 19.31 |

*vitro* effects and the $IC_{50}$s for doxycycline are dependent on the duration of incubation (8–10), on gas conditions, i.e., $O_2$ and $CO_2$ levels (11, 12), and on methodology, i.e., isotopic test versus immunoenzymatic or SYBR green test (13, 14). The incubation time is one of the conditions that interferes significantly with the $IC_{50}$s of antibiotics (10, 15). In the present study, the *in vitro* testing conditions (i.e., parasitemia, hematocrit, serum use, incubation time in the presence of doxycycline, isotopic test) were the same as those used in the previous works (3, 4, 7).

The distribution of doxycycline $IC_{50}$s for 620 *P. falciparum* isolates was analyzed by the Bayesian method to identify the presence of subpopulations with different levels of doxycycline chemosusceptibility as previously described for doxycycline (4) and for pyronaridine and piperaquine (16). Two distributions were

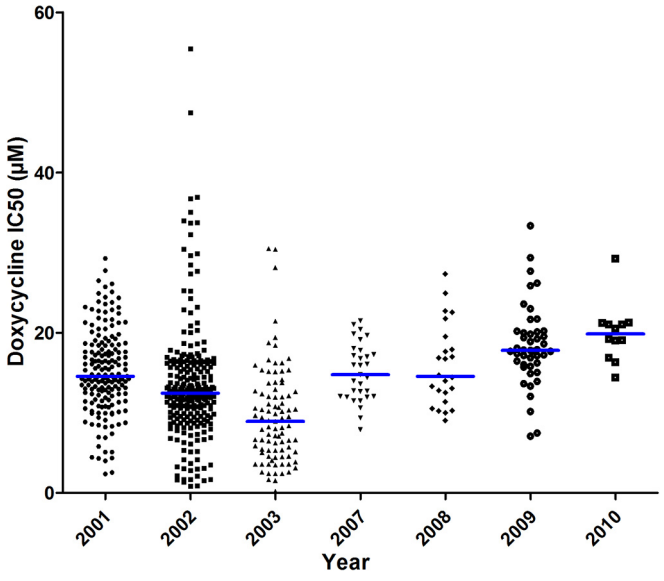


**FIG 1** Doxycycline 50% inhibitory concentrations ($IC_{50}$s) during the 2001-to-2010 period. The horizontal bars indicate the medians.

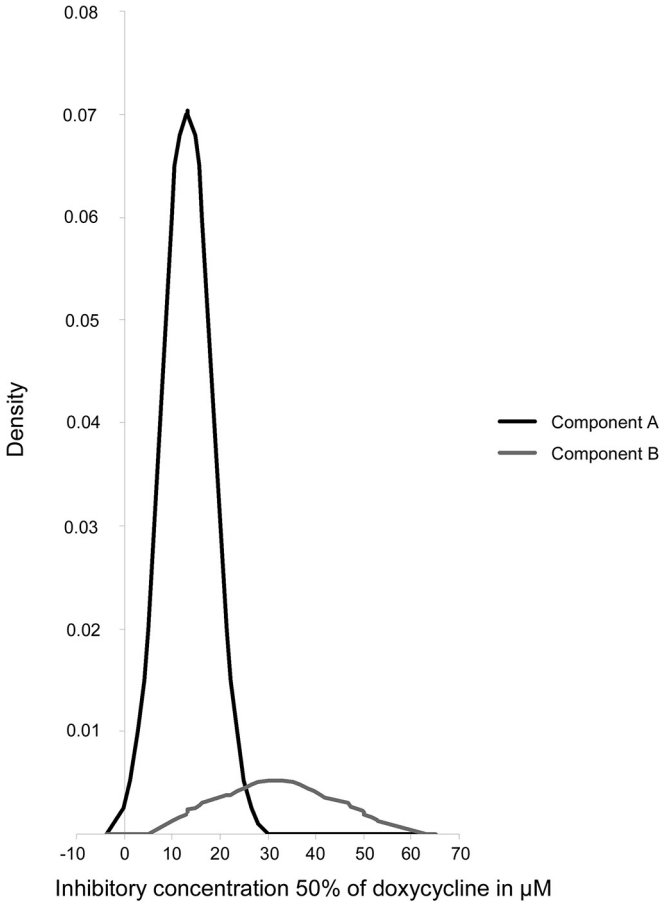


**FIG 2** Distribution of the doxycycline $IC_{50}$s of the 620 *Plasmodium falciparum* isolates from Thailand in the two-component mixture model (Bayesian mixture modeling approach).

**TABLE 2** Statistical analysis of *pftetQ* and *pfmdt* gene polymorphism in 89 *Plasmodium falciparum* isolates (Fisher's exact test)

| Gene copy no. and repeat frequency | No. (%) of isolates | | *P* value |
|---|---|---|---|
| | Component A | Component B | |
| No. of *pfmdt* copies | | | |
| >1 | 7 (12.1) | 4 (13.3) | |
| 1 | 51 (87.9) | 26 (86.7) | 1 |
| No. of *pTetQ* copies | | | |
| >1 | 1 (1.7) | 2 (6.7) | |
| 1 | 58 (98.3) | 28 (93.3) | 0.26 |
| No. of PfTetQ KYNNNN repeats | | | |
| <3 | 11 (18.6) | 7 (23.3) | |
| 3 | 48 (81.4) | 23 (76.7) | 0.59 |

identified with, respectively, a mean value (± standard deviation) of 13.15 ± 5.25 μM for phenotypic group A, including 590 isolates (95.3%), and a mean value of 31.60 ± 9.39 μM for phenotypic group B, including 30 isolates (4.7%) (Fig. 2). This differs from the three doxycycline phenotypes observed in *P. falciparum* African isolates under the same laboratory conditions (3, 4).

Two recent studies have demonstrated that there was an association between the *pfmdt* and *pftetQ* copy numbers and the level of doxycycline susceptibility in Africa (3, 4). In addition, in Kenya, Achieng et al. showed that isolates with one copy of *pfmdt* had a median $IC_{50}$ lower than those with two or more *pfmdt* copies (5). The quantification of *pfmdt* and *pftetQ* copy numbers of 59 *P. falciparum* Thai isolates randomly chosen from phenotypic group A with low or moderate doxycycline $IC_{50}$s (mean, 9.41 μM [95% confidence interval [CI], 3.61 to 15.21 μM]) and all 30 isolates from group B with high doxycycline $IC_{50}$s (mean, 29.16 μM [95% CI, 22.76 to 35.56 μM]) was performed by TaqMan real-time PCR under the same conditions as previously described for African isolates (3, 4, 17). Two isolates possessed two copies of *pftetQ* and one copy of *pfmdt*. Ten isolates had one copy of *pftetQ* and two copies of *pfmdt*. Only one isolate had two copies of both *pftetQ* and *pfmdt*. All isolates with two copies of *pfmdt* or *pftetQ* had one allelic family for each of the two genes (*msp1* and *msp2*) as determined by the use of the nested PCR strategy previously described (18), confirming that these infections were clonal. Mixed-clone infections could influence TaqMan real-time PCR readouts, potentially leading to false-positive gene copy number data.

In Thai isolates, there was no association between *pfmdt* or *pftetQ* copy numbers and the level of susceptibility to doxycycline (Table 2 and Table 3). This result differs from data from previous studies in African isolates but is similar to data from a recent study from Senegal that found no significant association between doxycycline *in vitro* susceptibility and increased copy numbers of *pftetQ* ($P = 0.08$) or *pfmdt* ($P = 0.07$) (17).

In addition, reduced *in vitro* doxycycline susceptibility was found associated with PfTetQ KYNNNN sequence polymorphism: a total of <3 KYNNNN motif repeats is predictive of *P. falciparum* parasites with resistance *in vitro*, with $IC_{50}$s of >35 μM (odds ratio of 15) (5). There was no association between doxycycline *in vitro* susceptibility and the number of PfTetQ KYNNNN repeats in Thai isolates (Table 2 and Table 3). This result differs from those obtained in Kenyan isolates (5). The difference in these data might be explained by the differences in the methods used for parasite $IC_{50}$ evaluation (isotopic assay versus SYBR green I-based assay), data interpretation, sample size, parasite genetic background, population structure, and much more.

These results may also indicate that the molecular mechanisms of resistance to doxycycline are more complex than anticipated. The overexpression of *pftetQ* or *pfmdt* and the PfTetQ KYNNNN sequence polymorphism could confer reduced *in vitro* susceptibility to doxycycline in association with other contributing determinants which could modulate the *in vitro* response to doxycycline. Some genes which encoded apicoplast proteins such as apicoplast ribosomal protein S10 (*arps10* gene; PF3D7_1460900.1) or ferrodoxin (*fd* gene; PF3D7_1318100), a key component of the apicoplast electron transport chain, might be involved in doxycycline resistance. These two genes might be also involved in *P. falciparum* artemisinin resistance (19). Thus, further studies are needed to better characterize the genetics of doxycycline resistance in *P. falciparum*.

**TABLE 3** Statistical analysis of the doxycycline $IC_{50}$s (in μM) based on the *pftetQ* and *pfmdt* copy numbers and PfTetQ KYNNNN repeat numbers in 89 *Plasmodium falciparum* isolates

| Parameter | Value | | | | | |
|---|---|---|---|---|---|---|
| | No. of *pftetQ* copies | | No. of *pfmdt* copies | | No. of PfTetQ KYNNNN repeats | |
| | 1 | >1 | 1 | >1 | <3 | 3 |
| No. of isolates | 86 | 3 | 77 | 11 | 18 | 71 |
| Minimum $IC_{50}$ | 2.17 | 15.20 | 2.17 | 2.58 | 3.03 | 2.17 |
| 25% percentile | 4.34 | 15.20 | 4.33 | 4.44 | 4.59 | 4.44 |
| Median $IC_{50}$ | 14.62 | 25.88 | 14.68 | 14.9 | 14.64 | 14.68 |
| 75% percentile | 24.15 | 29.38 | 25.11 | 25.88 | 27.08 | 23.73 |
| Maximum $IC_{50}$ | 55.44 | 29.38 | 55.44 | 36.92 | 36.92 | 55.44 |
| Mean | 15.66 | 23.49 | 15.85 | 16.49 | 16.99 | 15.65 |
| SD | 11.08 | 7.39 | 11.21 | 10.87 | 11.86 | 10.89 |
| SE | 1.20 | 4.27 | 1.28 | 3.28 | 2.79 | 1.29 |
| Lower 95% CI of mean | 13.28 | 5.14 | 13.30 | 9.19 | 13.92 | 14.87 |
| Upper 95% CI of mean | 18.03 | 41.84 | 18.39 | 23.79 | 20.71 | 16.43 |
| Mann-Whitney test *P* value | | 0.11 | | 0.87 | | 0.72 |

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We declare that we have no competing interests.

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