

Supplementary Information

Anti-folate combination therapies and their affect on the development of drug resistance in *Plasmodium vivax*

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Extended Methods

Study sites and sampling information

Isolates of *P. vivax* included in this study were collected from Yunnan, Hainan Island and Central China area (Anhui, Henan and Hubei provinces) in which over 90 percent of the country's malaria cases occur.

Yunnan province is located in the far southwest of the country and borders Burma, Laos, and Vietnam. Average annual rainfall in Yunnan ranges from 600 mm to 2,300 mm, with over half the rain occurring between June and August. Yunnan has one of the highest malaria burdens in China. The areas we selected in this study include Tengchong city, Gongshan and Fugong counties along the Nu River, where epidemiological features of malaria and history of drug use was recorded. *Anopheles minimus* and *An. dirus* are the main vectors and transmission is year-round with two peaks (July and September). From the middle 1960's, the pyrimethamine and sulfadoxine in combination were introduced as antimalarial prophylactic remedy in Yunnan. Until the early 1990s, use of SP combination was inactivated when the drug resistance was discovered by a local institute.

Central China area includes five malaria-endemic provinces, i.e. Henan, Anhui, Hubei, Jiangsu and Shandong. The area has a distinct seasonal climate characterized by humid summers and dry winters. The annual rainfall averages between 500–750mm across the region and the rainy season lasts from June to September. Malaria in Central China area can be classified as seasonally unstable and epidemic. *An. sinensis* and *An. anthropophagus* are the main vectors. We collected the *P. vivax* isolates in this study from the regions where *vivax* malaria were recently reemerged, including Lizhai and Maqiao

counties in Henan province, Yingshang, Huaiyuan, Woyang, Huaibei counties in Anhui province and Xiangyang city in Hubei province. In the early 1960s, pyrimethamine was introduced as an anti-recurrence medicine and prophylactic. The use of pyrimethamine was stopped in the middle 1980s and replaced by chloroquine and primaquine.

Hainan Island is located in Southern China. It is a relatively geographically isolated area hot throughout the year. The average annual precipitation is 1,500 to 2,000 mm and can be as high as 2,400 mm in central and eastern areas. The average relative humidity is around 80%. The major malaria vectors in this region are *An. dirus* and *An. minimus*.

Malaria transmission is perennial and fresh malaria cases are reported throughout the year. In the Island, *vivax*-endemic area mainly encompasses the regions including Sanya city, Dongfang and Ledong counties where we collected the samples. Pyrimethamine was introduced on Hainan Island in 1959, and then combined with sulfadoxine in 1967.

However the combination of antifolates were confined to several villiages in the southern and southwestern parts of island during 1968 to 1972. With the emergence of chloroquine-resistant and pyrimethamine-resistant *plasmodium* strains in the middle 1970s, piperazine was massively employed as alternative antimalarial agents to treat or prevent malarial infections in the island since 1979. In addition, the combination of both antifolate drugs plus either primaquine or artemisinin were used as a presumptive treatment and for chloroquine-resistant *falciparum* parasite treatment.

Table S1 Primers and profiles used for amplification of the *Pvdhfr* and *Pvdhps* gene

| Primers | Sequences | Annealing Temp(°C) | Product Size (bp) | Cycles | |
|----------|---------------------------------|-----------------------|----------------------|-----------------|-----------------|
| | | | | 1 st | 2 nd |
| POF | 5' CACCGCACAGTTGATTCCT 3' | 58 | 979 | 20 | |
| POR | 5' CCTCGGCGTTGTTCTTCT 3' | | | | |
| PBF | 5' CCC CAC CAC ATA ACG AAG 3' | 58 | 755 | | 25 |
| PAR | 5' CCC CAC CTT GCT GTA AAC C 3' | | | | |
| DHPS-F1 | 5'GATGGCGGTTTATTTGTCG 3' | 58 | 1009 | 18 | |
| DHPS-R1 | 5' GCTGATCTTTGTCTTGACG 3' | | | | |
| DHPS-F2* | 5'GCTGTGGAGAGGATGTTC 3' | 59 | 731 | | 22 |
| DHPS-R2* | 5' CCGCTCATCAGTCTGCAC 3' | | | | |
| PDR2 | 5'-AAGGAGAACCAGCAAGAC-3' | 59 | 3237 | 26 | |
| PDF2* | 5'-AAGCGTAGCGACAGAAG-3' | | | | |
| S4* | 5'-CAGGCGATGCTCTACGTT-3' | 60 | 3023 | | 26 |
| S1* | 5'-CGACAGAAGAACGCA-3' | | | | |
| S2* | 5'-TTCAGTGACGCTCGGATT-3' | | | | |
| S3* | 5'-TACCACGCTACCGAGT-3' | | | | |

Table S2 Microsatellite markers in the flanking regions of the *P. vivax dhfr* gene

| Microsatellite | Pos. from dhfr | Repeat unit sequence | Repeat Array |
|----------------|----------------|----------------------|--------------|
| u93 | -93kb | TA | 10 |
| u38 | -38kb | AT | 9 |
| u2 | -2.6kb | AAATC-AAATT-AAATG | 7-8-4 |
| d4 | +4.9kb | AT-ac-AT-g-TATG | 4-9-3 |
| d37 | +37kb | TA-c-AT | 5-5 |
| d94 | +94kb | CT | 8 |

Table S3 Primers and profiles used for amplification of the flanking microsatellites

| Primers | Sequences | Annealing Temp(°C) | Product Size (bp) | Cycles | | Size range (bp) |
|---------|-------------------------------|-----------------------|----------------------|-----------------|-----------------|--------------------|
| | | | | 1 st | 2 nd | |
| u93F4 | 5' TGTTTTACCCCTTCAGATA 3' | 50 | 267 | 15 | | |
| u93R3 | 5' CTCCTGCTTTGGTACAATAA 3' | | | | | |
| u93F | 5' GGTGAATAAGGGACCAAT 3' | 47 | 138 | 22 | | 132~154 |
| u93R | 5' CCCACATTTGTTGCTAA 3' | | | | | |
| u38F | 5'ACCCACGCACATCCGTA 3' | 55 | 307 | 25 | | |
| u38R2 | 5' TGGGCTATTCCGTCAATCAA 3' | | | | | |
| u38F | 5'ACCCACGCACATCCGTA 3' | 60 | 140 | 20 | | 146~160 |
| u38R | 5'GGGTGGGGGCATCTATTT 3' | | | | | |
| u2F8 | 5'G CTCCTCACAAACACATACAAAT 3' | 57 | 850 | 18 | | |
| u2R7 | 5'GCGGCTACACAGTGAAAAATA 3' | | | | | |
| u2.6F3 | 5' GGCTATACGAAGACAAAAC 3' | 52 | 300 | 25 | | 245~305 |
| u2.6R | 5' AACCCCTGACCGTTACAT 3' | | | | | |
| d4.9F0 | 5' GAGGGAACGGCTTTTCTT 3' | 52 | 179 | 15 | | |
| d4.9R | 5' AGTGGGCCAAACAAATA 3' | | | | | |
| d4.9F | 5' GGGCAAAGCTGAACAT 3' | 58 | 110 | 35 | | 108~122 |
| d4.9R | 5' AGTGGGCCAAACAAATA 3' | | | | | |
| d38F | 5' TGTGCTGTCTGGAGAATA 3' | 48 | 152 | 35 | | 148~160 |
| d38R | 5' CCACTTTTGGACACTAAA 3' | | | | | |
| d94F | 5' CGAGCAGGTTCTCATAGA 3' | 50 | 122 | 35 | | 116~140 |
| d94R | 5'CCCTAATTTGGCATGAAA 3' | | | | | |

Table S4 Expect heterozygosity of 6 polymorphic loci

| | u93 | u38 | u2 | d4 | d37 | d94 |
|--|-------------|-------------|-------------|-------------|-------------|-------------|
| Sensitive alleles | 0.729±0.018 | 0.772±0.020 | 0.764±0.014 | 0.725±0.029 | 0.757±0.018 | 0.807±0.022 |
| 117N alleles in Central China area | 0.812±0.012 | 0.675±0.035 | 0.251±0.061 | 0.615±0.030 | 0.728±0.025 | 0.670±0.037 |
| 58R/117N alleles in Hainan | 0.677±0.052 | 0.535±0.067 | 0.090±0.059 | 0.303±0.074 | 0.566±0.076 | 0.840±0.022 |
| 57I(L)/58R/61M/117T alleles in Yunnan | 0.742±0.026 | 0.692±0.033 | 0.606±0.033 | 0.274±0.052 | 0.568±0.039 | 0.823±0.027 |

Table S5 The entire *P.vivax* *pppk*-*dhps* of 158 isolates in Yunnan field isolates

| <i>dhps</i> | Intron1 | | <i>pppk</i> | Intron2 | | | Repetitive domain | | | | | | | frequency |
|-------------|---------|------|-------------|---------|-------|-------|-------------------------------|----------|----------|-------------------------------|----------|----------|----------|-----------|
| | 141G | 262A | | 205M | 2327G | 2389G | 2410C | 1 | 2 | 3 | 4 | 5 | 6 | |
| SAKA | G | A | M | G | G | C | GEAKLTN- GEGKLTN- GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 2 |
| SAKA | G | A | M | G | G | I | GEAKLTN- GEGKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 1 |
| SAKA | G | A | I | G | G | C | GEAKLTN- GEGKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 4 |
| SAKA | G | A | I | G | G | C | GEAKLTN- GEGKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 6 |
| SAKA | G | A | I | G | G | C | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 2 |
| SAKA | G | A | I | G | G | C | GEAKLTN- | GEGKLTN- | — | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 5 |
| SAKA | G | A | I | G | G | C | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 1 |
| SAKA | G | A | I | G | G | C | GEAKLTN- GEGKLTN- | GEGKLTN- | — | — | GDAKLTN- | GDSKLTN- | GEAKLTN- | 1 |
| SAKA | G | A | I | A | G | I | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 1 |
| SAKA | G | A | I | A | G | I | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 2 |
| SAKA | G | I | I | G | G | I | GEAKLTN- GEGKLTN- GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- GDAKLTN- GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 2 |
| SGKA | G | A | I | G | G | I | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 5 |
| SGKA | I | A | I | G | G | I | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 3 |
| SGKA | G | A | I | G | G | C | GEAKLTN- GEGKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- GDSKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 2 |
| SGKA | G | I | I | G | G | C | GEAKLTN- GEGKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GDSKLTN- | 1 |
| SGKA | G | I | I | G | G | C | GEAKLTN- | GEGKLTN- | — | — | GDAKLTN- | GDSKLTN- | GEAKLTN- | 3 |
| SGKA | G | I | I | A | G | I | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GDSKLTN- | 3 |

| | | | | | | | | | | | | | | |
|-------------|---|----------|----------|----------|----------|----------|-------------------|----------|----------|----------|----------|----------|----------|----|
| AGKA | G | <u>I</u> | <u>I</u> | G | G | C | GEAKLTN- GEGKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 1 |
| AGKA | G | A | <u>I</u> | G | G | C | GEAKLTN- GEGKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 18 |
| AGKA | G | A | <u>I</u> | G | G | C | GEAKLTN- | GEGKLTN- | — | — | GDAKLTN- | GDSKLTN- | GEVKLTN- | 2 |
| SGKG | G | <u>I</u> | <u>I</u> | G | G | C | GEAKLTN- | GEGKLTN- | — | — | GDAKLTN- | GDSKLTN- | GEVKLTN- | 3 |
| SGKG | G | A | <u>I</u> | <u>A</u> | G | <u>I</u> | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 1 |
| SGKG | G | <u>I</u> | <u>I</u> | G | G | <u>I</u> | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 1 |
| SGKG | G | <u>I</u> | <u>I</u> | G | <u>I</u> | <u>I</u> | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 8 |
| SGKG | G | <u>I</u> | <u>I</u> | <u>A</u> | G | <u>I</u> | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 60 |
| AGKG | G | <u>I</u> | <u>I</u> | <u>A</u> | G | <u>I</u> | GEAKLTN- GEGKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 3 |
| AGKG | G | <u>I</u> | <u>I</u> | <u>A</u> | G | <u>I</u> | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 1 |
| AGKG | G | A | <u>I</u> | <u>A</u> | G | <u>I</u> | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 3 |
| AGKG | G | A | <u>I</u> | G | G | C | GEAKLTN- GEGKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 10 |
| SGMG | G | <u>I</u> | <u>I</u> | <u>A</u> | G | <u>I</u> | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 1 |
| SGMG | G | <u>I</u> | <u>I</u> | G | <u>I</u> | <u>I</u> | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 1 |
| SGTG | G | <u>I</u> | <u>I</u> | <u>A</u> | G | <u>I</u> | GEAKLTN- | GEGKLTN- | GEAKLTN- | GEGKLTN- | GDAKLTN- | GDSKLTN- | GEAKLTN- | 1 |

Legend:

The table S5 shows details of the mutations in coding regions, introns and indels in the *pvpppk-dhps* gene. The first column is referred to as single letter amino acid abbreviation of codons 382, 383, 512 and 553 in *dhps*. The frequency of each haplotype is in the last column. The mutant amino acids in bold are underlined.

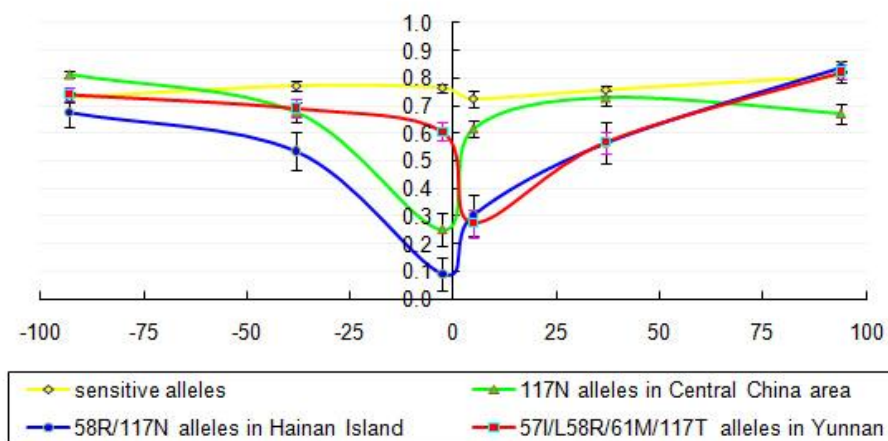
Table S6 Prevalence of *pvdhfr* and *pvdhps* mutant alleles in *P. vivax* isolates from Thailand and Yunnan

| | <i>dhfr</i> | | <i>dhps</i> | |
|-------------------|-------------|--------------|-------------|--------------|
| | Thailand | Yunnan,China | Thailand | Yunnan,China |
| wild type | 1.90% | 8.64% | 1.90% | 17.79% |
| Single mutant | 0.60% | 7.27% | 26.30% | 12.50% |
| Double mutants | 35.60% | 11.81% | 60.60% | 60.10% |
| Triple mutants | 2.50% | 11.80% | 9.50% | 9.61% |
| Quadruple mutants | 59.40% | 60.45% | 1.90% | — |

Legend:

The table S6 shows the frequency of *dhfr* and *dhps* genotypes. Quadruple mutant alleles in *dhfr* (57I/L+58R+61M+117T) and double mutant alleles in *dhps* (383G+553G) were observed frequently in isolates collected from Thailand and Yunnan, where both under long-term SP selection pressures. So we analyzed linkage disequilibrium for the Thailand isolates.

Figure S1.Expected heterozygosity around different *dhfr* alleles.



Legend:

The microsatellite markers are ordered along the x-axis and He (± 1 s.d.) plotted against position on the chromosomes relative to *dhfr*. The allele types with over 40 samples from a single region are plotted. Microsatellite variability is reduced to varying degrees around the *dhfr* locus in the resistant alleles.