CHAPTER V
RESULTS

1. Assessment of sensitivity of *P. vivax* isolates *in vitro*

Our first goal was to assess in short-term culture, the sensitivities of *P. vivax* field isolates to three inhibitors of DHFR, i.e. pyrimethamine, chlorcycloguanil (active metabolite of chlorproguanil), and the experimental antifolate WR99210, and as well as two inhibitors of DHPS, i.e. sulfadoxine and dapsone. A total of 32 *P. vivax* isolates were assessed for the sensitivity to these drugs by comparing the maturation of rings to schizonts in the absence and presence of a range of drug concentrations. It was noted that the assessment was not performed in all isolates due to insufficient parasites volume. All stages of asexual development (rings, trophozoites and schizonts) were observed in the peripheral blood and the initial parasitemia in the isolates varied between 1,280 and 18,000 /µl blood **(Table 4)**. For each drug, a wide range of IC_{50} values was obtained. The IC_{50} values are depicted in **Figure 16** according to their $\log_{10}$ values and the geometric means and median values and ranges are presented in **Table 5.** The range of IC_{50} values was markedly wide for pyrimethamine (almost four orders of magnitude). Despite the wide range, the mean values do show consistent differences in the response to all of the five drugs. First, considering all of the isolates, WR99210 inhibited growth most potently; not even the highest IC_{50} values exceeded 600 nM. Second, chlorcycloguanil was substantially less effective than either pyrimethamine or WR99210, even among the most sensitive isolates. Finally, the IC_{50} values measured for the two sulfa drugs were extremely high, while dapsone was more potent than sulfadoxine, as has been observed when similar measurements were made in *P. falciparum* (Mberu *et al.*, 2002).

Table 4 The IC_{50} values obtained for short term *in vitro* susceptibility assessment of 32 *P. vivax* field isolates to pyrimethamine, chlorcycloguanil, WR99210, sulfadoxine and dapsone

Isolate	IC_{50}(nM)					Parasiteamia (/µl blood)
	PYR	CCG	WR	SD	DDS	
MSPV002	1.57	246.65	49.32	505.77	880.09	1720
MSPV004	24.64	ND	ND	ND	ND	1280
MSPV007	3.14	ND	ND	ND	ND	2160
MSPV008	123.14	ND	ND	ND	ND	1880
MSPV010	8.53	ND	ND	ND	2065.38	3680
MSPV012	18.93	2634.57	125.12	5832.97	1378.06	4000
MSPV013	70.42	412.66	62.48	7995.52	1156.90	2840
MSPV015	34.35	352.15	42.62	4704.54	1817.03	3000
MSPV016	9.49	623.33	70.22	4987.53	1130.73	5120
MSPV017	125.17	961.29	68.54	3076.18	1245.08	3200
MSPV020	114.42	324.01	142.60	6425.02	1696.00	2240
MSPV021	20.96	1183.22	86.79	1827.56	2461.35	1280
MSPV022	6.41	662.99	25.62	2652.23	1884.71	2460
MSPV023	1715.41	520.56	62.71	5059.72	3527.09	1280
MSPV024	52.65	164.26	28.17	4099.44	1823.84	1800
MSPV030	617.86	545.72	25.08	9059.04	1207.21	12900
MSPV031	38.15	667.96	143.28	1281.01	3667.71	1600
MSPV032	1857.94	656.95	343.89	3012.67	1801.22	2640
MSPV033	14.22	363.37	86.35	639.25	349.31	4000
MSPV035	4.58	529.87	39.61	321.47	763.52	18000
MSPV036	97.14	842.35	95.95	2049.63	973.42	5520
MSPV037	244.44	302.93	51.06	703.89	867.83	4800
MSPV038	365.52	3541.59	380.69	2508.35	2881.70	2560
MSPV040	126.53	459.26	46.63	1050.23	1173.02	11040
MSPV041	5463.04	2112.92	374.34	2317.39	3353.22	5760
MSPV042	96.87	675.50	357.50	1048.56	3705.70	5200
MSPV046	2846.98	1893.67	118.60	3518.47	3274.67	7200
MSPV048	1324.04	1322.58	54.50	2915.17	2379.77	2880
MSPV050	3752.06	1548.40	114.46	7999.26	2324.16	1880
MSPV051	80.07	661.85	178.82	2641.33	2273.82	4880
MSPV052	106.23	2007.00	588.56	615.85	943.28	5880
MSPV057	31.07	6279.33	144.23	5972.60	1179.11	7920
GeoMean	85.20	783.69	95.10	2423.56	1624.77	
Medium	88.47	662.42	86.57	2783.70	1801.22	
N	32	28	28	28	29	

Note: ND=Not done, PYR=pyrimethamine, CCG=chlorcycloguanil, WR=WR99210, SD=sulfadoxine, and DDS=dapsone

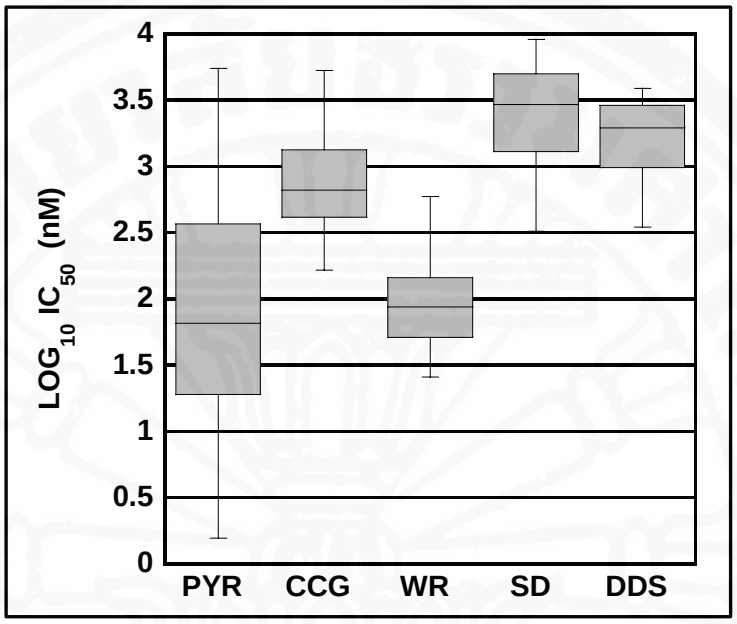

Figure 16 Box plot of the $\log_{10}$ of the IC_{50} values measured in the short term *in vitro* culture system in *P. vivax* field isolates from Mae Sot, Thailand. The ranges of values for pyrimethamine (PYR), chlorcycloguanil (CCG), WR99210 (WR), sulfadoxine (SD), and dapsone (DDS) are depicted. The box indicates the central 50% of values and the median of all values, with the 95% confidence interval indicated.

Table 5 Geometric means, arithmetic median and range of the IC_{50} values for each of the drugs in the short term *in vitro* culture system. Data are presented as number (N), geometric mean, and median (range) values.

DRUG	N	Geometric mean IC_{50} (nM)	Median (range) IC_{50} (nM)
Pyrimethamine	**32**	**85**	**88 (1.6 - 5463)**
Chlorcycloguanil	**28**	**784**	**662 (164 - 6279)**
WR99210	**28**	**95**	**87 (26 - 589)**
Sulfadoxine	**28**	**2424**	**2784 (321 - 9059)**
Dapsone	**29**	**1625**	**1801 (349 - 3668)**

2. Detection of mutations in the *Pvdhfr* and *Pvdhps* genes

The sensitivity of *P. falciparum* isolates to antifolates *in vitro* depends principally upon the genotypes of the *dhfr* and *dhps* genes in the parasite (Cowman *et al.*, 1988; Hyde, 2002; Mberu *et al.*, 2002; Peterson *et al.*, 1988; Triglia *et al.*, 1997). To test whether this correlation is valid for *P. vivax*, we sequenced *dhfr* and *dhps* from all isolates. We identified five different *Pvdhfr* alleles and four *Pvdhps* alleles **(Table 6)**. The frequencies of the genotypes are presented in **Table 7.** Twenty six of the 32 isolates carried quadruple mutant alleles of *Pvdhfr*; one allele has been observed in isolates from several sites previously (F57L/ S58R/T61M/S117T) (Hastings *et al.*, 2005; Imwong *et al.*, 2001; Imwong *et al.*, 2003; Kaur *et al.*, 2006; Tjitra *et al.*, 2002), and the other is an allele that differs at amino acid 57 (F57I/S58R/T61M/S117T) and has been observed previously only in Thailand (Imwong *et al.*, 2003). In addition, there were four triple mutants (S58R/T61M/S117T, K49C/S58R/S117N) and two isolates with the double mutant genotype commonly seen (S58R/S117N) (Kaur *et al.*, 2006; Kocken *et al.*, 2006; Menegon *et al.*, 2006; Valecha *et al.*, 2006). All isolates carried a valine at DHPS residue 585; this residue has been hypothesized to account for *P. vivax's* low susceptibility to sulfadoxine (Korsinczky *et al.*, 2004). In addition to 585V, two isolates were mutant solely at *dhps* residue 383. Twenty four isolates carried double mutant alleles of *Pvdhps* at residues 383 and 553 (A383G/A553G) and six isolates carried an additional mutation at residue 382 (S382A/A383G/A553G, S382C/A383G/A553G). No wild type allele for either gene was identified.

Table 6 Genotyping of *Pvdhfr* and *Pvdhps* in a total of 32 *P. vivax* isolates collected from Mae Sot District, Tak Province.

Isolate	DHFR Genotype	DHPS Genotype
MSPV002	S58R/S117N	A383G/A553G
MSPV004	F57I/ S58R/T61M/S117T	A383G/A553G
MSPV007	F57I/ S58R/T61M/S117T	A383G/A553G
MSPV008	F57I/ S58R/T61M/S117T	A383G/A553G
MSPV010	F57I/ S58R/T61M/S117T	S382C/A383G/A553G
MSPV012	F57I/ S58R/T61M/S117T	A383G/A553G
MSPV013	F57I/ S58R/T61M/S117T	S382A/A383G/A553G
MSPV015	F57I/ S58R/T61M/S117T	A383G/A553G
MSPV016	F57I/ S58R/T61M/S117T	S382A/A383G/A553G
MSPV017	F57I/ S58R/T61M/S117T	S382A/A383G/A553G
MSPV020	K49C/S58R/S117N	A383G
MSPV021	F57I/ S58R/T61M/S117T	A383G/A553G
MSPV022	F57L/ S58R/T61M/S117T	A383G/A553G
MSPV023	F57I/ S58R/T61M/S117T	A383G/A553G
MSPV024	S58R/T61M/S117T	A383G/A553G
MSPV030	F57I/ S58R/T61M/S117T	A383G/A553G
MSPV031	F57I/ S58R/T61M/S117T	S382C/A383G/A553G
MSPV032	F57I/ S58R/T61M/S117T	A383G/A553G
MSPV033	S58R/T61M/S117T	A383G/A553G
MSPV035	F57L/ S58R/T61M/S117T	A383G/A553G
MSPV036	F57L/ S58R/T61M/S117T	A383G/A553G
MSPV037	S58R/S117N	A383G
MSPV038	F57L/ S58R/T61M/S117T	A383G/A553G
MSPV040	F57L/ S58R/T61M/S117T	A383G/A553G
MSPV041	F57L/ S58R/T61M/S117T	A383G/A553G
MSPV042	F57L/ S58R/T61M/S117T	A383G/A553G
MSPV046	F57I/ S58R/T61M/S117T	A383G/A553G
MSPV048	F57L/ S58R/T61M/S117T	S382A/A383G/A553G
MSPV050	F57I/ S58R/T61M/S117T	A383G/A553G
MSPV051	S58R/T61M/S117T	A383G/A553G
MSPV052	F57L/ S58R/T61M/S117T	A383G/A553G
MSPV057	F57I/ S58R/T61M/S117T	A383G/A553G

Table 7 Allele frequencies of *Pvdhfr* and *Pvdhps in P. vivax* isolates from Mae Sot District, Tak Province.

Genotype of *dhfr* allele	**Genotype of *dhps* allele**	**Frequency (%)**
S58R/S117N	A383G/A553G	1 (3.1%)
	A383G	1(3.1%)
K49C/S58R/S117N	A383G	1(3.1%)
S58R/T61M/S117T	A383G/A553G	3 (9.4%)
F57L/ S58R/T61M/S117T	A383G/A553G	8 (25%)
	S382A/A383G/A553G	1 (3.1%)
F57I/ S58R/T61M/S117T	A383G/A553G	12 (37.5%)
	S382A/A383G/A553G	3(9.4%)
	S382C/A383G/A553G	2(6.3%)

Note: All isolates carried *Pvdhps* 585V.

3. Association between sensitivity in short term *in vitro* culture system and *Pvdhfr* genotypes

Our primary goal was to correlate the genotypes of each isolate with the sensitivity of parasite isolates to each drug in the short term culture system. **Table 8** summarizes the sensitivities (IC_{50} values) of *P. vivax* (N=32) to pyrimethamine, chlorcycloguanil, WR99210, sulfadoxine and dapsone, in relation to their *pvdhfr* and *pvdhps* genotypes. It is noteworthy that, isolates carrying the same *dhfr* and *dhps* alleles still showed wide variation in their IC_{50} values for all drugs. For example, among isolates carrying quadruple mutant allele (F57L/S58R/T61M/S117T), the IC_{50} values for pyrimethamine varied from 4 to more than 5,000 nM and for the F57I/S58R/T61M/S117T allele, from 3 to more than 3000 nM. However, a trend of increasing mean IC_{50} values was observed with increasing number of *Pvdhfr* mutations from double to quadruple. The mean IC_{50} values for both pyrimethamine and chlorcycloguanil of the isolates with three or fewer mutations were significantly different from those carrying a quadruple mutant *dhfr* allele (pyrimethamine, p=0.02, and chlorcycloguanil, p=0.002). In contrast, the sensitivity to WR99210 in the two groups were comparable (p=0.1).

The table output is arranged in ascending order of the IC_{50} value for pyrimethamine. The range of IC_{50} values observed with other drugs was also wide, but no absolute correlation between sensitivity to pyrimethamine and chlorcycloguanil or WR99210 was found. On the other hand, it appears that chlorcycloguanil is less effective than pyrimethamine, although this observe was not reach statistical significance. Moreover, WR99210 retained its activity even against parasites that carry the quadruple mutant alleles of *Pvdhfr*.

As expected, the IC_{50} values for both sulfadoxine and dapsone were markedly high. This reflects both the relative low activity of this class of drugs when used alone against *Plasmodia* (Mberu *et al.*, 2000). Furthermore, all of the isolates were found to carry *dhps* alleles with 585V, as well as at least one other mutation in analogous with that reported by Korsinczky and colleagues (Korsinczky *et al.*, 2004). Sulfa drugs inhibit DHPS, but not DHFR enzyme, therefore one would not expect that the *dhfr* allele in an isolate would affect the response to these drugs, and that is what we observed. When we stratified the

isolates according to their *dhps* genotypes and compared the activities to sulfadoxine and dapsone, no difference in the IC_{50} values was observed in the groups.

Table 8 Relationship between *Pvdhfr- Pvdhps* genotypes in 32 *P. vivax* field isolates and susceptibility to pyrimethamine, chlorcycloguanil, WR99210, sulfadoxine and dapsone, as determined by short term *in vitro* culture. Data are presented as arithmatic mean values of the IC_{50} values and standard deviation.

Genotype		IC_{50} value (nM)				
Pvdhfr	*Pvdhps*	*PYR*	*CCG*	*WR*	*SD*	*DDS*
58R/117N	383G/553G	1.5	246	49	505	880
	383G	244	302	51	703	867
Mean ± SD		123 ± 17	274± 39	50± 10	604± 14	874± 90
58R/61M/117T	383G/553G	14	164	86	639	349
	383G/553G	52	164	28	4099	1823
	383G/553G	80	661	178	2641	2273
Mean ± SD		49 ± 33	330 ± 87	97 ±75	2460 ±17	1482 ± 10
49C/58R/117N	383G	114	324	107	6425	1096
57L/58R/61M/117T	383G/553G	4	529	39	321	763
	383G/553G	6	662	25	2652	1884
	383G/553G	96	675	357	1048	3705
	383G/553G	97	842	96	2050	973
	383G/553G	106	2007	588	615	943
	383G/553G	126	459	46	1050	1173
	383G/553G	365	3541	380	2508	2881
	382A/383G/553G	1324	1322	54	2915	2379
	383G/553G	5463	2112	374	2317	3353
Mean ± SD		843 ± 18	1350±10	218±21	1719±97	2006±11
57I/58R/61M/117T	383G/553G	3	ND	ND	ND	ND
	382C/383G/553G	8	ND	ND	ND	2066
	382A/383G/553G	9	523	70	4987	715
	383G/553G	18	2634	125	5832	1378
	383G/553G	21	1183	87	1828	2461
	383G/553G	24	ND	ND	ND	ND
	383G/553G	31	6279	144	5972	1179
	383G/553G	34	352	42	4704	2390
	382C/383G/553G	38	667	143	1281	2469
	382A/383G/553G	70	412	62	7995	1156
	383G/553G	123	ND	ND	ND	ND
	382A/383G/553G	125	961	58	3076	ND
	383G/553G	617	545	25	9059	1207
	383G/553G	1715	520	62	5059	3527
	383G/553G	1857	656	343	3012	3870
	383G/553G	2846	1893	118	3518	3274
	383G/553G	3752	1548	114	7999	2324
Mean ± SD		670 ± 11	1398±16	107± 77	4948± 24	2155±99

PYR=pyrimethamine, CCG=chlorcycloguanil, WR=WR99210, SD=sulfadoxine, DDS=dapsone

4. Clonality of the isolates

One possible explanation for the wide variation in the IC_{50} values among isolates with the same *Pvdhfr* genotype might be that the isolate contains more than one parasite clone. None of the sequences of the *Pvdhfr* or *Pvdhps* genes showed evidence for polymorphism within these loci. However, we did assess the clonality of the infection by assaying the size of the insert in the *PvMSP-3*gene. Three size classes were observed, type A (1.9 Kb), B (1.5 Kb), and C (1.1 Kb). All isolates carried the class A repeat except for one isolate that carried size class B and three isolates that carried size class C.

After digestion with *Hha* I, the PCR-restriction fragment length polymorphism pattern suggested that some isolates showed patterns indicative of polyclonal infection. Polyclonality was distinguished when the summed size of the fragments digested with *Hha* I exceeded the size of the uncut PCR product. We assumed that if the sum of the fragments exceeded the size of the uncut fragment, the isolate was polyclonal. By that criterion, nine isolates were polyclonal and none of these isolates showed a minority allele at *Pvdhfr* or *Pvdhps*. Moreover, the IC_{50} values that were far from the expected median for that *Pvdhfr* genotype were observed in apparently monoclonal isolates.

5. Determination of drug sensitivity in yeast expression system

Due to the absence of long term culture system for *P. vivax*, we have turned to a heterologous yeast system to compare the efficacy of DHFR inhibitors against various alleles of *Pvdhf* (Hastings *et al.*, 2005; Hastings and Sibley, 2002). To compare directly the sensitivities of these field isolates in the short term culture and that in the yeast system, the *P. vivax* DHFR domain from each of the 5 alleles, including the wild-type control allele (de Pecoulas *et al.*, 1998a) was amplified, and cloned into the yeast shuttle vector by homologous recombination and transformed into yeast that lacked endogenous DHFR activity. The IC_{50} values for each strain against pyrimethamine, WR99210 and chlorcycloguanil were measured by the relative growth of the yeast *in vitro*. The yeast were grown in a range of drug concentrations up to 5×10^{-4} M and the IC_{50} value for each allele was calculated. **Figure 17** summarizes representative data. These IC_{50} values were then used to assess the relative resistance level compared to the wild-type allele **(Table 9)**.

All mutant alleles showed increased resistance to pyrimethamine and chlorcycloguanil by more than 50 fold when compared with wild-type allele. In contrast, all mutant alleles were relatively sensitive to WR99210 (relative resistance 1.3-83), particularly the S58R/S117N alleles. As expected, the values are consistent with those measured previously for the same *Pvdhfr* alleles (Hastings *et al.*, 2005; Hastings and Sibley, 2002). The current data also support the usefulness of the yeast expression system in assaying sensitivity of *P. vivax* to antifolates. In addition, chlorcycloguanil was demonstrated to be particularly ineffective against all but the wild type allele.

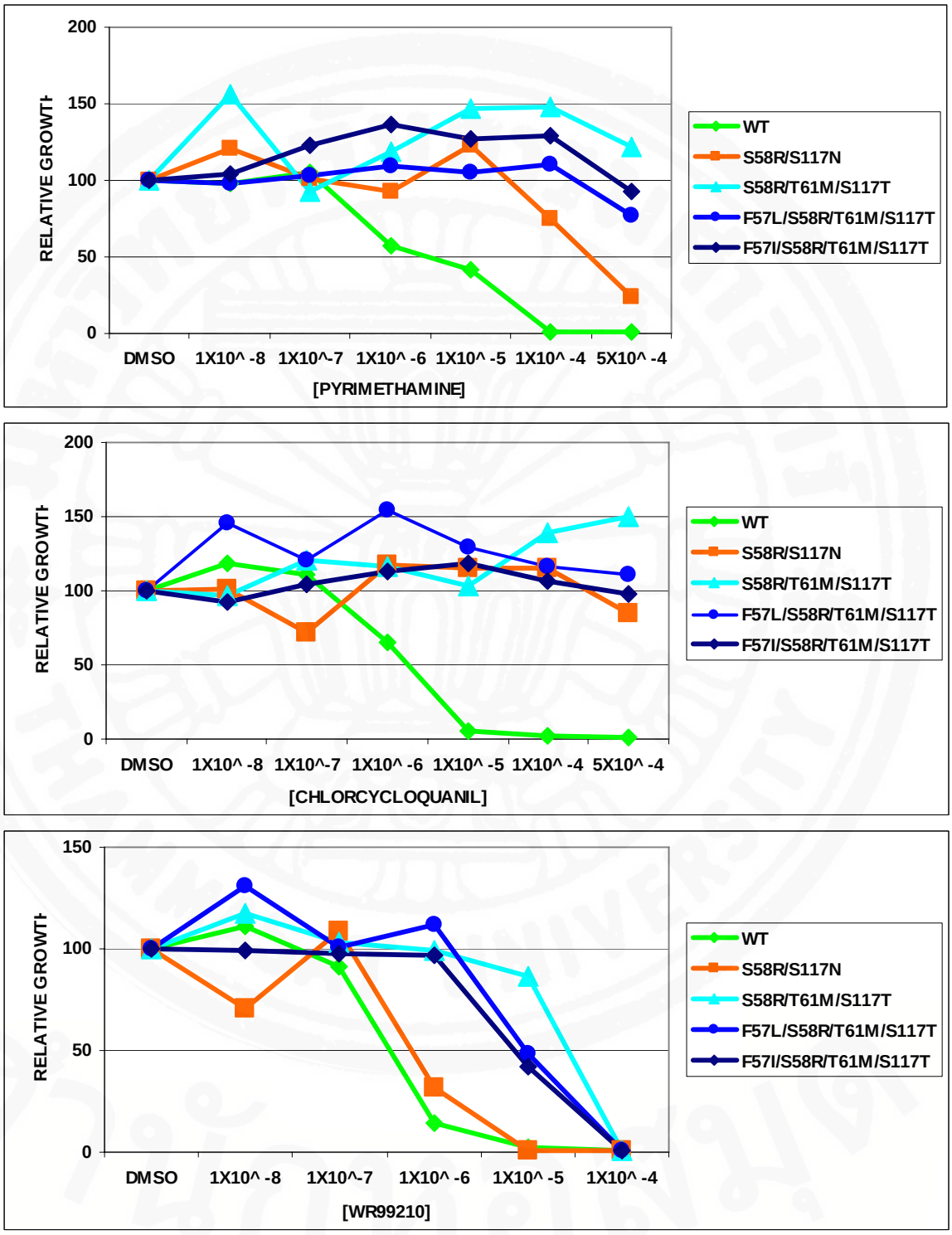

Figure 17 Sensitivity of yeast to antifolate drugs dependent on various *Pvdhfr* alleles to pyrimethamine, chlorcycloguanil and WR99210

Table 9 IC_{50} values (mean±SD) for yeast dependent upon each *Pvdhfr* allele and the resistance relative to wild-type (RR). Strains in which no IC_{50} value could be measured are listed as greater than the highest concentration tested (5×10^{-4} M).

Genotype	Pyrimethamine		Chlorcycloguanil		WR99210	
	IC_{50} ±SD (nM)	**RR**	**IC_{50} ±SD (nM)**	**RR**	**IC_{50} ±SD (nM)**	**RR**
S58 /S117 (WT)	5196 ± 258	**1**	3282 ± 730	**1**	582 ± 9.9	**1**
58R/117N	281,602 ± 85610	**54**	>500,000	**>152**	787 ± 29	**1.3**
58R/61M/117T	>500,000	**>96**	>500,000	**>152**	48215 ± 1073	**83**
57L/58R/61M/117T	>500,000	**>96**	>500,000	**>152**	9776 ± 35.4	**17**
57I/58R/61M/117T	>500,000	**>96**	>500,000	**>152**	8636 ± 574.5	**15**

6. Geographical distribution of *Pvdhfr* and *Pvdhps* genes

6.1 Detection of mutations in the *Pvdhfr* gene

A total 160 isolates of *P. vivax* infected blood samples were collected from different geographical regions of malaria endemic areas along the international borders of Thailand. Their *dhfr* and *dhps* were amplified by nested PCR and their sequences were determined. **Figure 18** shows the sequences of *Pvdhfr* and *Pvdhps* obtained in comparison with the wild-type sequences (GenBank accession no. X98123 and AY186730, respectively). Sequence comparison revealed that three isolates were wild-type allele for *pvdhfr*; the remaining *pvdhfr* alleles showed mutant genotypes. Among these mutant isolates, the most common *Pvdhfr* alleles were quadruple (59.4%) and double mutant (35.6%). Only one isolate carried single mutation at residue 117, and four isolates carried triple mutations. The frequency of *Pvdhfr* genotypes of the identified alleles are listed in **Table 10.** Double mutation (58R/117N) was observed frequently in isolates collected from Thai-Cambodian (Chanthaburi and Trad) and Thai-Malaysian (Yala) borders. Two alleles of quadruple mutations, 57L/58R/61M/117T and 57I/58R/61M/117T were found commonly in isolates collected along the Thai-Myanmar border. Single mutation at residue 117 (S117T) was observed in only one isolate collected from Mae Hong Son and two allele of triple mutation (58R/61M/117T and 49G/58R/117N) were found in Tak. **Figure 19** shows the location of each sampling areas and the pie charts indicate the proportions of *Pvdhfr* alleles present in each location.

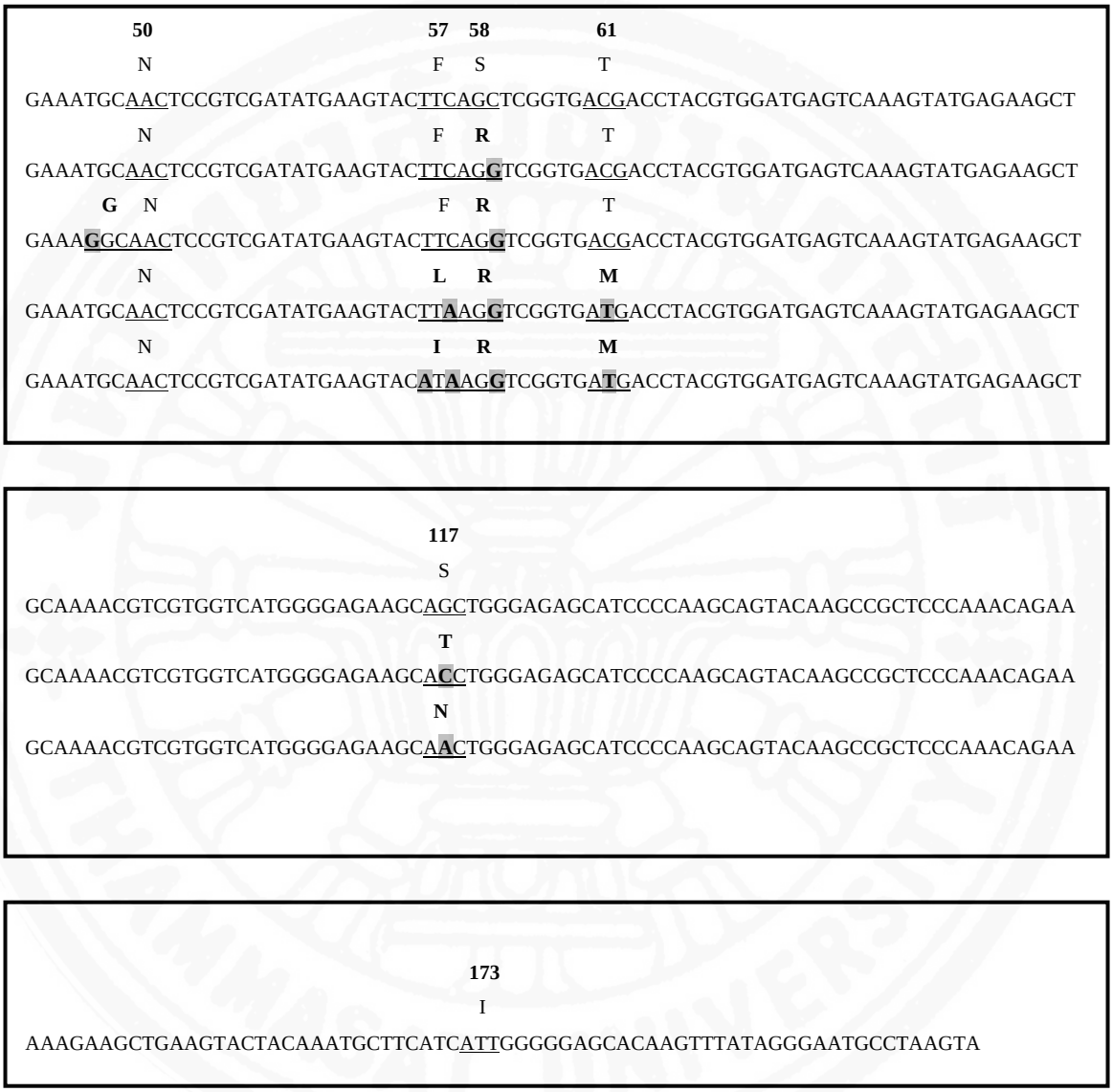

Figure 18 The sequences of *Pvdhfr* obtained in comparison with the wild-type sequence at indicated residues (Highlight and bold letters are mutate residues).

Table 10 Allele frequencies of *Pvdhfr* mutation in 160 *P. vivax* isolates from Thailand

Allele	Amino acid position, *Pvdhfr*					Number (%)
	49	57	58	61	117	
Wild-type	C	F	S	T	S	3 (1.9%)
Single	C	F	S	T	**T**	1 (0.6%)
Double	C	F	**R**	T	**N**	57 (35.6%)
Triple 1	C	F	**R**	**M**	**T**	3 (1.9%)
Triple 2	**G**	F	**R**	T	**N**	1 (0.6%)
Quadruple 1	C	**L**	**R**	**M**	**T**	18 (11.3%)
Quadruple 2	C	**I**	**R**	**M**	**T**	77 (48.1%)

Note: Grey boxes indicated mutate animo acid

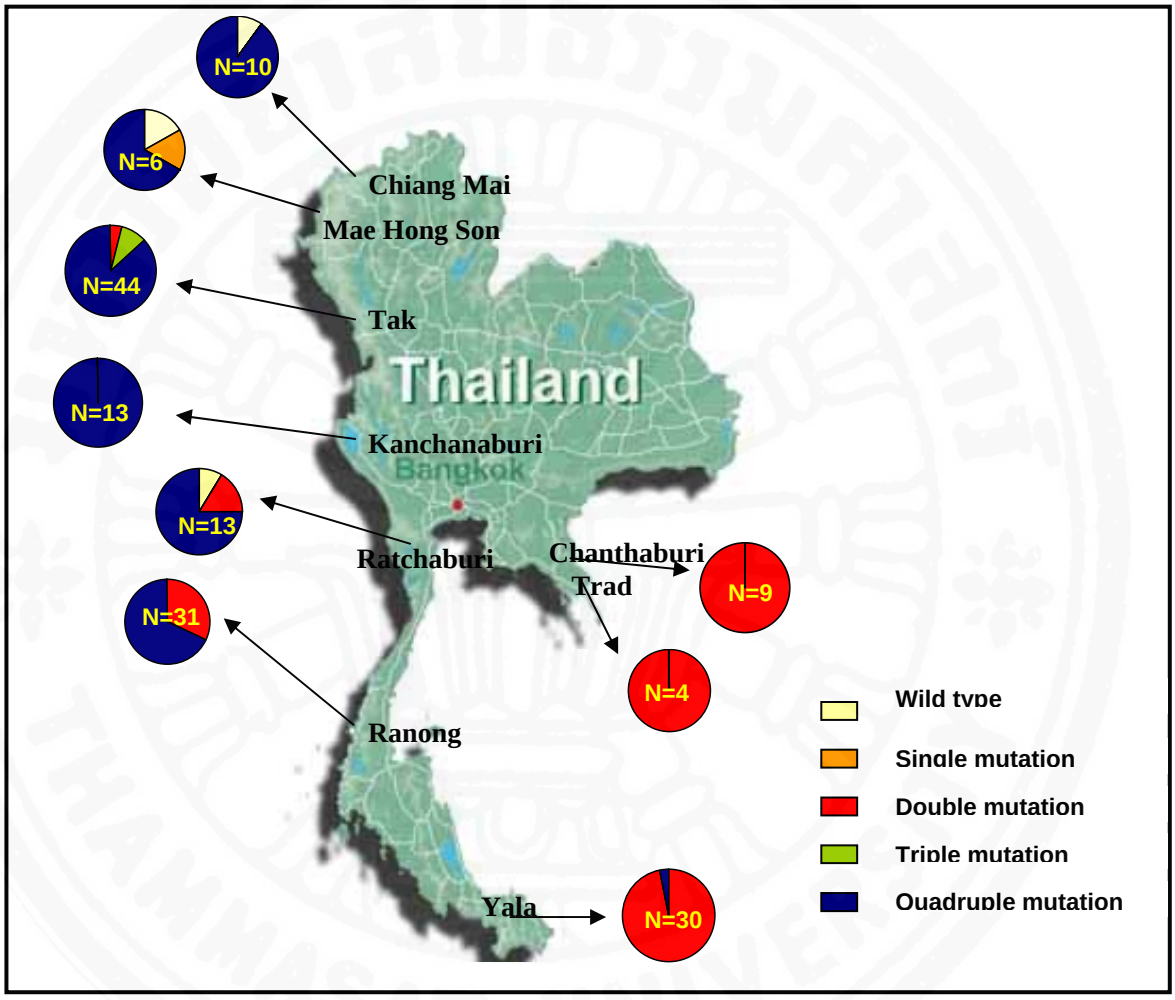

Figure 19 Map of different originating *P. vivax* samples derived from Thailand. The pie charts show the proportions of *Pvdhfr* alleles

6.2 Detection of mutations in the *Pvdhps* gene

All identified *Pvdhps* genotypes were shown in **Figure 20**. The frequency of *Pvdhps* genotypes of the identified alleles are listed in **Table 11.** The most prevalent *Pvdhps* allele was double mutant allele (383G/553G) (60%), followed by single mutant (26.9%). Only 2 isolates (1.3%) present gene sequence identical to wild type sequence for *pvdhfr*. Fifteen isolates carried triple mutation and 3 isolates carried quadruple mutation. Novel mutations that have not been previously identified at codon 512 (K512M, K512E, K512T) was found in 7 isolates collected from Chiang Mai, Mae Hong Son and Kanchanaburi Province. The proportions of *Pvdhps* alleles distributed in different geographical areas of Thailand are shown in **Figure 21**.

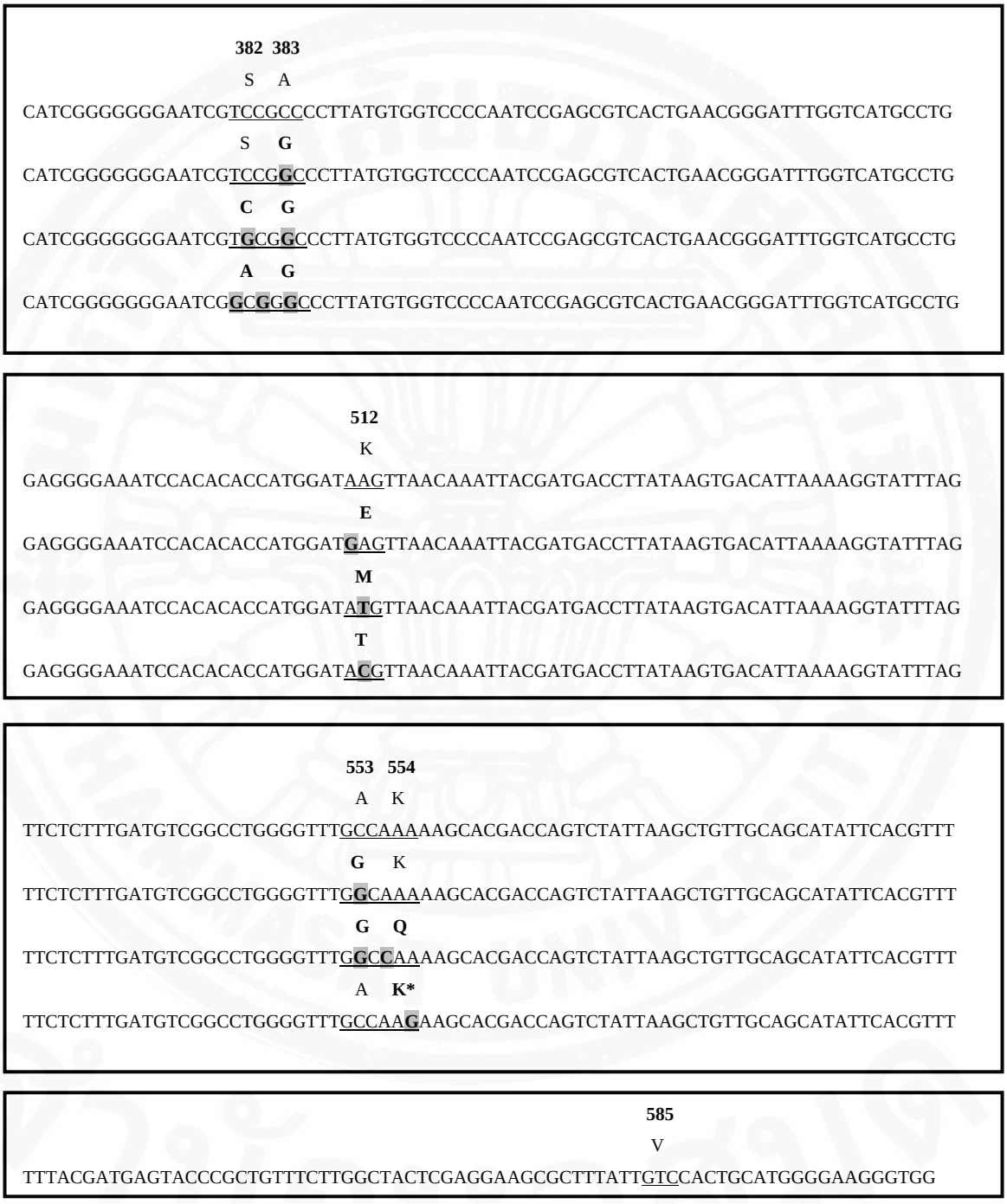

Figure 20 The sequences of *Pvdhps* obtained in comparison with the wild-type sequence at indicated residues (Highlight and bold letters are mutate residues).

Table 11 Prevalence of *Pvdhps* mutant alleles in 160 *P. vivax* isolates from Thailand, and the corresponding codon in *P. falciparum dhps*.

Grey boxes indicate mutant amino acids. The # allele had a synonymous change at codon 554 K=aaa, K* = aag.

Allele	***Species***	**Codon with synonymous change**					**Number (%)**
	Pfdhps	**S436F/A**	**A437G**	**K540E**	**A581G**	**A613S**	
	Pvdhps	**382**	**383**	**512**	**553**	**585**	
Wild-type		S	A	K	A	V	2 (1.3%)
	#	S	A	K*	A	V	1 (0.6%)
Single		S	**G**	K	A	V	42 (26.3%)
Double		S	**G**	K	**G**	V	96 (60.0%)
		S	**G**	K	**C**	V	1 (0.6%)
Triple		**A**	**G**	K	**G**	V	8 (5.0%)
		C	**G**	K	**G**	V	3 (1.9%)
		S	**G**	**M**	**G**	V	2 (1.3%)
		S	**G**	**T**	**G**	V	2 (1.3%)
Quadruple		**C**	**G**	**E**	**G**	V	2 (1.3%)
		A	**G**	**M**	**G**	V	1 (0.6%)

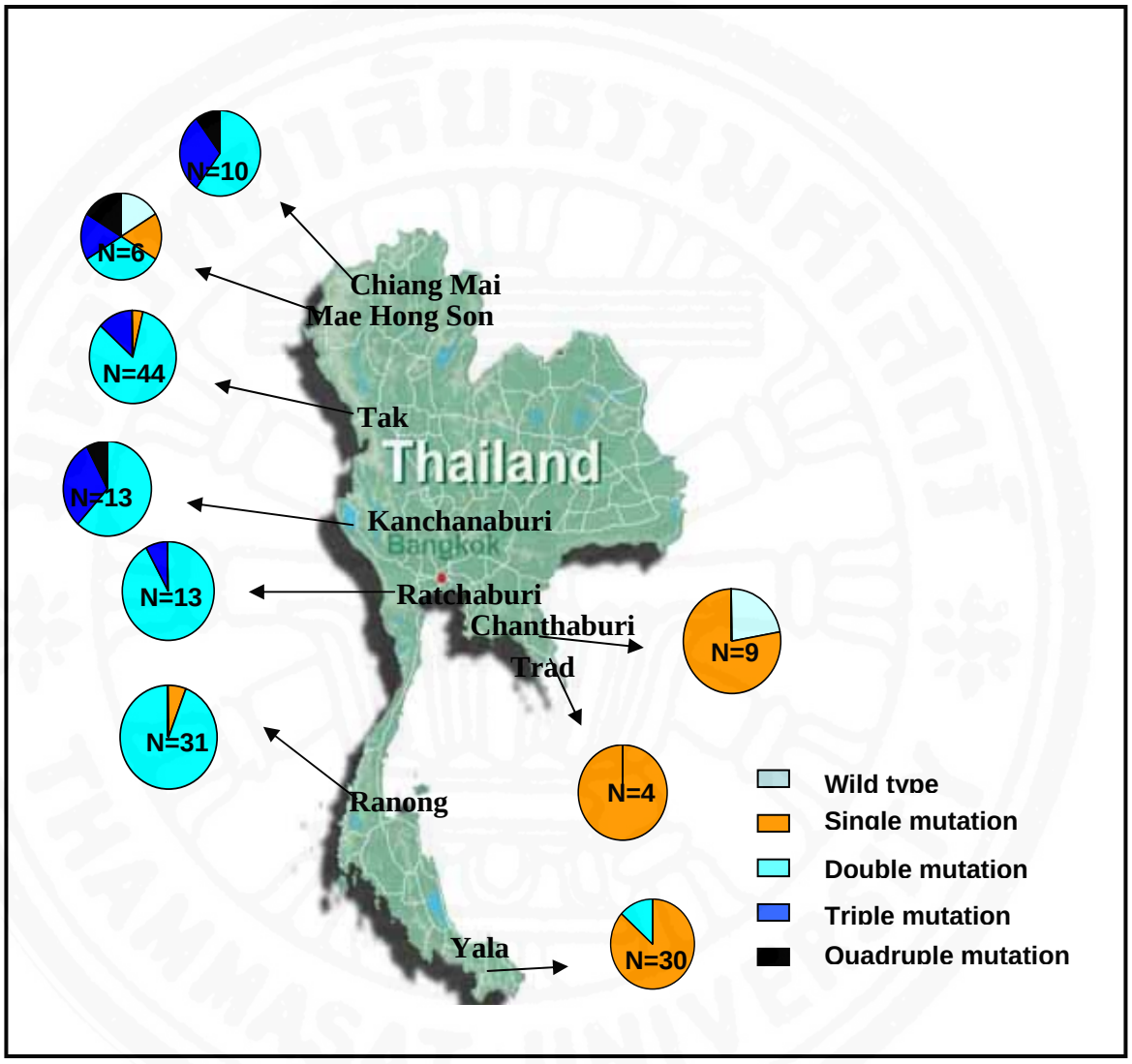

Figure 21 Map of different originating *P. vivax* samples derived from Thailand. The pie charts show the proportions of *Pvdhps* alleles.

6.3 Distribution of *Pvdhfr* and *Pvdhps* alleles from different geographical areas of Thailand

Our samples taken from various geographical areas showed allelic diversity of *Pvdhfr* and *Pvdhps* genes. There were 7 and 11 alleles identified for *Pvdhfr* and *Pvdhps*, respectively, with a total of 20 different haplotypes of combined *dhfr* and *dhps* mutation. Distribution of these alleles from different geographical malaria endemic areas is shown in **Table 12.** The most prevalent haplotype (36.3%) carried quadruple mutant allele (57I/58R/61M/117T) of *dhfr* combined with double mutant allele (383G/553G) of *dhps*, followed by double mutant allele (58R/117N) of *dhfr* combined with single mutant allele (383G) of *dhps* (24.2%). No combined wild-type of both genes was observed. Three isolates contained single or double mutant *dhps* coupled with *dhfr* wild-type, whereas two isolates contained double mutant *dhfr* coupled with wild-type *dhps*. Ninety-seven isolates (60.6%) contained at least quintuple mutant allele of *dhfr-dhps* combinations.

Table 12 Allelic frequencies of *Pvdhfr* and *Pvdhps* from 160 *P. vivax* isolates collected form Chiang Mai (CM), Mae Hong Son (MH), Tak (T), Kanchanaburi (K), Ratchaburi (RB), Ranong (RN), Chanthaburi (CH), Trad (BR) and Yala (YL) Province.

Genotype of *dhfr* allele	Genotype of *dhps* allele	Areas									Frequency (%)
		Thai-Myanmar						Thai-Cambodian		Thai-Malaysian	
		CM	MH	T	K	RB	RN	CH	BR	YL	
Wild type	383G		1								1 (0.6)
	383G/553G	1				1					2 (1.3)
117T	383G/553G		1								1 (0.6)
58R/117N	Wild type							2			2 (1.3)
	383G			1			1	7	4	26	39 (24.4)
	383G/553G			1		3	9			2	15 (9.4)
	383G/553C									1	1 (0.6)
58R/61M/117T	383G/553G			3							3 (1.9)
49G/58R/117N	383G			1							1 (0.6)
57L/58R/61M/117T	383G/553G			16			1				17 (10.6)
	382A/383G/553G			1							1 (0.6)
57I/58R/61M/117T	383G						1				1 (0.6)
	554K*		1								1 (0.6)
	383G/553G	5	1	16	8	8	19			1	58 (36.3)
	382A/383G/553G	1		3	2	1					7 (4.4)
	382C/383G/553G	1		2							3 (1.9)
	383G/512T/553G	1	1								2 (1.3)
	383G/512M/553G				2						2 (1.3)
	382A/383G/512M/553G				1						1 (0.6)
	382C/383G/512E/553G	1	1								2 (1.3)
Total		10	6	44	13	13	31	9	4	30	160

K*=aag

7. Determination of drug sensitivity in yeast expression system

We amplified the *P. vivax* DHFR domain and cloned into the yeast shuttle vector by homologous recombination. Six different alleles were expressed in yeast cells that lack endogenous DHFR activity, including wild type allele, single, double, triple and two quadruple mutant alleles. Transformed yeast were grown overnight in complete medium lacking dTMP and 0-5 x 10^{-4} M pyrimethamine, chlorcycloguanil or WR99210. The response of each *Pvdhfr* allele to pyrimethamine, chlorcycloguanil and WR99210 were measured by its relative growth. The IC_{50} value for each allele was calculated to measure the relative resistance compared to the wild-type allele **(Figure 22 and Table 13)**. Triple and two quadruple mutants showed 80-fold more resistant to pyrimethamine and chlorcycloguanil compared with wild-type allele; the growth of yeast cells exceeded the maximum concentration of pyrimethamine and chlorcycloguanil. In contrast, single mutant allele was relatively sensitive to all tested drugs. Double mutant allele was also sensitive to WR99210 but relatively resistant to pyrimethamine and chlorcycloguanil. All mutant alleles were sensitive to WR99210 (relative resistance 1.2-19.3) except triple mutant allele (relative resistance 95.2).

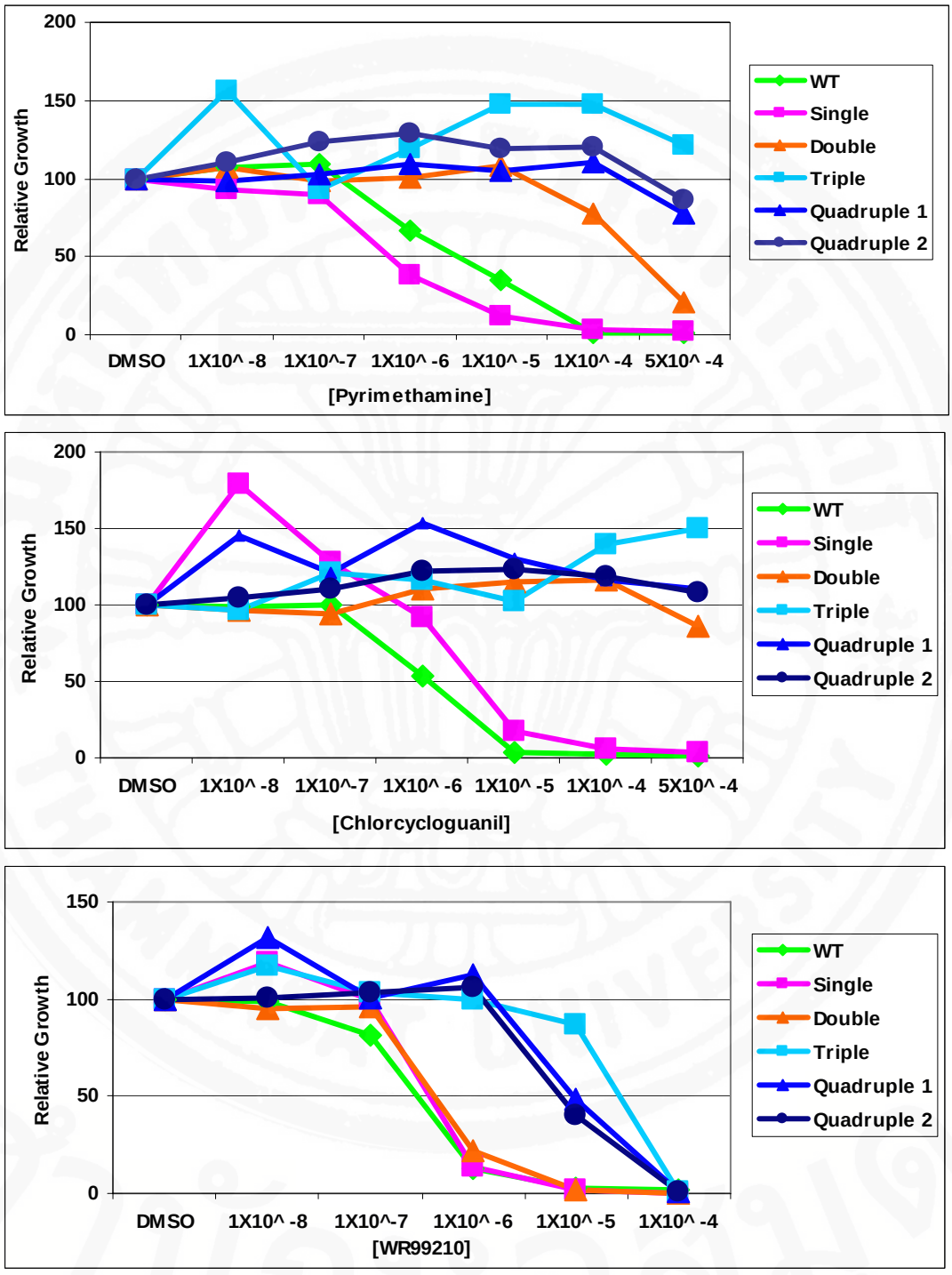

Fig 22 Relationship between *in vitro* sensitivity based on yeast expression system (expressed as IC_{50} values) and patterns of *Pvdhfr* allelic variants

Table 13. Association of *in vitro* sensitivity mean±SD value based on yeast expression system (expressed as IC_{50} values), patterns of *Pvdhfr* allelic variants, and the resistance relative to wild-type (RR)

Genotype	Pyrimethamine		Chlorcycloguanil		WR99210	
	IC_{50}±SD (nM)	**RR**	**IC_{50}±SD (nM)**	**RR**	**IC_{50}±SD (nM)**	**RR**
Wild-type	5600.8±731.3	**1**	1495.0±1679	**1**	506.4±72.9	**1**
S117T	1312±3221	**0.23**	2243±5018	**1.5**	586.5±201.2	**1.2**
S58R/S117N	237175±139673	**42.4**	>500000	**>334**	617.6±198	**1.2**
S58R/T61M/S117T	>500,000	**>89**	>500,000	**>334**	48215±1037	**95.2**
F57L/S58R/T61M/S117T	>500,000	**>89**	>500,000	**>334**	9750.6±37.4	**19.3**
F57I/S58R/T61M/S117T	>500,000	**>89**	>500,000	**>334**	8721.5±584.2	**17.2**