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▣ BRIEF COMMUNICATION

Genetic Polymorphisms in *Plasmodium vivax* Dihydrofolate Reductase and Dihydropteroate Synthase in Isolates from the Philippines, Bangladesh, and Nepal

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Abstract: Genetic polymorphisms of *pvdhfr* and *pvdhps* genes of *Plasmodium vivax* were investigated in 83 blood samples collected from patients in the Philippines, Bangladesh, and Nepal. The SNP-haplotypes of the *pvdhfr* gene at the amino acid positions 13, 33, 57, 58, 61, 117, and 173, and that of the *pvdhps* gene at the positions 383 and 553 were analyzed by nested PCR-RFLP. Results suggest diverse polymorphic patterns of *pvdhfr* alone as well as the combination patterns with *pvdhps* mutant alleles in *P. vivax* isolates collected from the 3 endemic countries in Asia. All samples carried mutant combination alleles of *pvdhfr* and *pvdhps*. The most prevalent combination alleles found in samples from the Philippines and Bangladesh were triple mutant *pvdhfr* combined with single mutant *pvdhps* allele and triple mutant *pvdhfr* combined with double wild-type *pvdhps* alleles, respectively. Those collected from Nepal were quadruple mutant *pvdhfr* combined with double wild-type *pvdhps* alleles. New alternative antifolate drugs which are effective against sulfadoxine-pyrimethamine (SP)-resistant *P. vivax* are required.

Key words: *Plasmodium vivax*, *Plasmodium vivax* dihydrofolate reductase (*pvdhfr*), *Plasmodium vivax* dihydropteroate synthase (*pvdhps*), malaria, PCR-RFLP, sulfadoxine-pyrimethamine (SP)

Plasmodium vivax, the causative agent of relapsing benign tertian malaria, constitutes the second most common cause of malaria in the world with more than 80 million clinical cases annually [1]. In Nepal, *P. vivax* is the predominant malaria species, with the prevalence rate of about 80% of the total malaria cases [2]. Drug resistance monitoring was initiated in 1978 where both in vivo and in vitro studies were conducted with primary focus on *P. falciparum* resistance in Jhapa and Banke districts [2]. Chloroquine-resistant *P. falciparum* was first reported in the country in 1984 and subsequently resistance was spread to other districts. In 1996-1997, sulfadoxine-pyrimethamine (SP) that replaced chloroquine lost its efficacy. In 2000, the late treatment failure rate of *P. falciparum* cases treated with SP was found to be 57% [2]. For Bangladesh and the Philippines, prevalence rates of *P. vivax* infection were relatively lower (13 and 25%, respectively) [3]. Chloroquine resistance was first detected in 1970 in the bordering districts of Bangladesh, i.e., Mymensingh district and Chaklapunji Tea Estate in Habigonj district. Subsequently, the prevalence rates (RII+RIII) were increased from 10% in 1979 to 45% in 1987, to 57% in 1992 [4]. The molecular targets of action of sulfadoxine and pyrimethamine are dihydropteroate synthase (dhps) and dihydrofolate reductase (dhfr) enzymes, respectively. SP-resistance is determined by specific point mutations in these 2 parasite genes that cause alterations of key amino acid residues in the active sites of these enzymes and thus reduction in the affinity of the enzyme for the drug [5,6].

In most Asian countries, *P. vivax* infections are rarely treated with SP, but *P. vivax* isolates are exposed to SP during treatment of *P. falciparum* with mixed infections with *P. vivax* [7-10].

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This has caused a progressive selection of SP-resistant alleles in *P. vivax* isolates [11]. The prevalence of *P. vivax* dihydrofolate reductase (*pvdhfr*) and dihydropteroate synthase (*pvdhps*) mutations have been reported from several malaria endemic countries in Asia including Thailand [12,13], Indonesia [14], Nepal [15], India [16], Afghanistan [17], Iran [18], Papua New Guinea [19], Madagascar [20], and the Philippines [23]. For Nepal, previous reports on *pvdhfr* mutations were identified at S58R (68%, n=43) followed by S117N/T (54%, n=35) and F57L (11%, n=7). For the Philippines, 1 pattern of *pvdhfr* combination alleles were found, double (66.7%) mutant allele. The most prevalent *pvdhfr* and *pvdhps* combination alleles were double mutant *pvdhfr* 58R/117N alleles combined with single mutant *pvdhps* 383G allele (50.0%) and combined with single wild type *pvdhps* A383 allele (50.0%). In this study, the diversity of *pvdhfr* and *pvdhps* mutant alleles in *P. vivax* isolates collected during 2005-2011 from the Philippines, Bangladesh, and Nepal were investigated. The study is the first report of the polymorphic frequency and pattern of *pvdhfr* and *pvdhps* in Bangladesh and first report of the polymorphic frequency and pattern of *pvdhps* in Nepal.

A total of 83 blood samples with mono-infection of *P. vivax* were collected from patients attending healthcare facilities in the Philippines in 2005 (33 samples), Bangladesh in 2010 (31 samples), and Nepal in 2011 (19 samples). Approval of the study protocol was obtained from the Ethics Committees of Ministry of Public Health of the Philippines, Bangladesh, and Nepal. Finger-prick blood (200-300 µl) samples were collected onto a filter paper (Whatman No. 3). The dried filter paper samples were stored in the plastic zip bags until analysis. Giemsa-stained thin and thick blood smears were prepared for qualitative and quantitative examination of *P. vivax* parasitemia under a light microscope. Parasite genomic DNA was extracted from individual dried blood spots on the filter paper using a QIAamp DNA extraction mini-kit (QIAGEN, Valencia, California, USA) and stored at -20˚C until use.

The primers and amplification conditions used for genotyping of *pvdhfr* were according to that previously reported with modifications [15,21]. All primers and restriction enzymes were obtained from Fermentas (Waltham, Massachusetts, USA) and Biolabs (Ipswich, Massachusetts, USA), respectively. *Pvdhfr*-OF and *pvdhfr*-OR primers were used for amplification of the first reaction of *pvdhfr*. PCR cycling condition was as follow: denaturation at 95˚C for 5 min, followed by 30 cycles of 95˚C for 30 sec, 64˚C for 30 sec, 72˚C for 30 sec, and 72˚C for 5 min.

Amino acid codons 13, 33, 58, and 61

The amplification of point mutations at amino acid codons 13, 33, 58, and 61 was performed using *pvdhfr*-13F and *pvdhfr*-13R primers. The PCR cycling condition was as follows: denaturation at 95˚C for 5 min, followed by 25 cycles of 95˚C for 30 sec, 66˚C for 30 sec, 72˚C for 30 sec, and 72˚C for 5 min. The PCR products were digested with restriction enzymes Hae III, Cfr42I (Sac II), Alu I, and Tsp45 I to detect the point mutations I13L, P33L, S58R, and T61M, respectively, and thereafter separated on 3% agarose gel.

Amino acid codons 57 and 173

The amplification of point mutations at amino acid codons 57 and 173 was performed using *pvdhfr*-F57 and *pvdhfr*-NR primers. The PCR cycling condition was as follows: denaturation at 95˚C for 5 min, followed by 25 cycles of 95˚C for 30 sec, 66˚C for 30 sec, 72˚C for 30 sec, and 72˚C for 5 min. The PCR products were digested with restriction enzymes Xmn I and Eco130I (Sty I) to detect the point mutations F57I/L and I173L, respectively, and thereafter separated on 3% agarose gel.

Amino acid codons 57 and 117

The amplification of point mutations at amino acid codons 57 and 117 were performed using *pvdhfr*-OF and *pvdhfr*-NR primers. The PCR cycling condition was as follows: denaturation at 95˚C for 5 min, followed by 25 cycles of 95˚C for 30 sec, 66˚C for 30 sec, 72˚C for 30 sec, and 72˚C for 5 min. The PCR products were digested with restriction enzymes BsrG I, Pvu II, Bsr I, and BstN I to detect the point mutations F57I and S117T/N, respectively, and thereafter separated on 3% agarose gel.

The primers and amplification conditions used for genotyping of *pvdhps* were according to that previously reported with modifications [15,18,21]. All primers and restriction enzymes were obtained from Fermentas and Biolabs, respectively. The amplification for first reaction of *pvdhps* was used *pvdhps*-OF and *pvdhps*-OR primers. The PCR cycling condition was as follows: denaturation at 95˚C for 5 min, followed by 25 cycles of 95˚C for 1 min, 58˚C for 2 min, 72˚C for 2 min, and 72˚C for 5 min.

Amino acid codons 383 and 553

The amplification of point mutations at amino acid codons 383 and 553 were performed using *pvdhps*-NF and *pvdhps*-NR primers, and *pvdhps*-553OF and *pvdhps*-NR primers, respectively. The PCR cycling condition was as follows: denaturation at 95˚C for 5 min, followed by 25 cycles of 95˚C for 1 min, 50˚C

Table 1. The frequencies and patterns of *pvdhfr* and *pvdhps* single nucleotide polymorphisms in 83 *P. vivax* isolates (bold letters indicate mutant amino acids)

Gene	Amino acid position	SNPs	No. of isolates (%) Philippines (n=33)	No. of isolates (%) Bangladesh (n=31)	No. of isolates (%) Nepal (n=19)
Pvdhfr	13	I (wild-type)	33 (100.0)	31 (100.0)	19 (100.0)
		L (mutant)	0 (0.0)	0 (0.0)	0 (0.0)
	33	P (wild-type)	33 (100.0)	19 (61.3)	6 (31.6)
		L (mutant)	0 (0.0)[a]	12 (38.7)	13 (68.4)
	57	F (wild-type)	33 (100.0)	21 (67.7)	19 (100.0)
		I (mutant)	0 (0.0)	0 (0.0)	0 (0.0)
		L (mutant)	0 (0.0)	10 (32.3)[a]	0 (0.0)
	58	S (wild-type)	0 (0.0)	4 (12.9)	10 (52.6)
		R (mutant)	33 (100.0)	26 (83.9)	9 (47.4)[a]
		S/R (mutant)	0 (0.0)	1 (3.2)	0 (0.0)
	61	T (wild-type)	6 (18.2)	5 (16.1)	0 (0.0)
		M (mutant)	27 (81.8)	26 (83.9)	19 (100.0)
	117	S (wild-type)	0 (0.0)	7 (22.6)	10 (52.6)
		N (mutant)	32 (97.0)[a]	14 (45.2)	8 (42.1)
		T (mutant)	1 (3.0)	8 (25.8)	0 (0.0)
		N/T (mutant)	0 (0.0)	2 (6.4)	1 (5.3)
	173	I (wild-type)	33 (100.0)	31 (100.0)	19 (100.0)
		L (mutant)	0 (0.0)	0 (0.0)	0 (0.0)
Pvdhps	383	A (wild-type)	33 (100.0)	31 (100.0)	19 (100.0)
		G (mutant)	0 (0.0)	0 (0.0)	0 (0.0)
	553	A (wild-type)	0 (0.0)	31 (100.0)	19 (100.0)
		G (mutant)	33 (100.0)[a]	0 (0.0)	0 (0.0)

[a]Statistically significant difference from samples collected from the other 2 countries ($P<0.01$; chi-square test).

for 2 min, 72˚C for 2 min, and 72˚C for 5 min. The PCR products were digested with restriction enzymes Msp I and Msc I to detect the point mutations at positions A383G and A553G, respectively, and separated on 3% agarose gel.

Statistical analysis was performed using the SPSS statistical package (version 11.5 SPSS Inc, Chicago, Illinois, USA). The chi-square test was used to determine the difference in prevalence rates of *pvdhfr* and *pvdhps* mutant allele (s) of each SNP in *P. vivax* isolates collected from the 3 countries in Asia. Statistical significance level was set at $P=0.05$.

The diversity of mutations in *pvdhfr* and *pvdhps* genes of 83 *P. vivax* isolates collected during 2005-2011 from the Philippines, Bangladesh, and Nepal were investigated in this study. Results from the present study suggest diverse polymorphic patterns of *pvdhfr* alone as well as the combination patterns with *pvdhps* mutant alleles in *P. vivax* isolates collected from the 3 endemic countries in Asia. The polymorphic patterns of *pvdhfr* and *pvdhps* genes were highly diverse (Table 1). For *pvdhfr*, the wild-type I13 and I173 alleles were detected in all samples from the Philippines, Bangladesh, and Nepal. Three mutant alleles, i.e., 58R, 61M, and 117N were observed in samples collected from the Philippines with frequencies ranging from 81.8 to 100%. Eight mutant alleles at 5 amino acid residues, i.e., 33L, 57L, 58R, 58SR, 61M, 117N, 117T, and 117NT were detected in samples from Bangladesh at varying frequencies (32.3-87.1%). Five mutant alleles at 4 amino acid residues, i.e., 33L, 58R, 61M, 117N, and 117NT were observed in samples collected from Nepal with frequencies ranging from 47.4-100%. Significant differences in frequencies of the 4 *pvdhfr* mutations (33L, 57L, 58R, and 117N) were observed in samples from the 3 Asian counties. For *pvdhps*, all samples collected from the 3 countries carried wild-type A383 allele. The mutation at 553G allele was found in all samples collected from the Philippines, but not in samples from Bangladesh and Nepal.

In the previous study published in 2011 [15], double mutant *pvdhfr* allele was commonly detected in *P. vivax* isolates in Nepal, but the triple and quadruple *pvdhfr* mutant haplotypes which are associated with a high level of in vivo pyrimethamine resistance were not found. In this study, in addition to single (21.0%), double (31.6%), triple (10.6%), and quadruple (36.8%, 33L/58R/61M/117N) mutant alleles, *pvdhfr* mutant alleles were found in the 2011 Nepal isolates, and all isolates possessed the mutant 61M allele. No isolate carried any mutant allele of *pvdhps*, suggesting an increased level of pyri-

Table 2. Distribution of *pvdhfr* and *pvdhps* combination alleles in 83 *P. vivax* isolates included in the analyses (bold letters indicate mutant amino acids)

Pvdhps		*Pvdhfr*							Philippines (33)	Bangladesh (31)	Nepal (19)
A383G	A553G	I13L	P33L	F57I/L	S58R	T61M	S117T/N	I173L			
A	G	I	P	F	R	T	N	I	6	0	0
A	G	I	P	F	R	M	N	I	26	0	0
A	G	I	P	F	R	M	T	I	1	0	0
A	A	I	P	F	S	M	S	I	0	0	4
A	A	I	P	F	S	M	N	I	0	2	0
A	A	I	P	F	R	M	S	I	0	1	0
A	A	I	P	F	R	M	T	I	0	1	0
A	A	I	P	F	R	M	N	I	0	10	1
A	A	I	P	F	R	M	S,N	I	0	1	1
A	A	I	P	L	R	M	S	I	0	1	0
A	A	I	P	L	R	T	T	I	0	3	0
A	A	I	L	F	S	M	S	I	0	1	6
A	A	I	L	F	R	M	N	I	0	2	7
A	A	I	L	F	R	M	T	I	0	1	0
A	A	I	L	F	R	M	S,N	I	0	1	0
A	A	I	L	F	S,R	M	T	I	0	1	0
A	A	I	L	L	S	M	S	I	0	1	0
A	A	I	L	L	R	M	S	I	0	3	0
A	A	I	L	L	R	T	T	I	0	2	0
Total									33	31	19

methamine resistance, but not sulfadoxine in Nepal in 2011.

Only 1 pattern of *pvdhfr* allele, i.e., double (66.7%, 58R/117N) mutant allele and 2 patterns of *pvdhps* alleles (50% 383G and 50% A383), was previously reported in the isolates from the Philippines in 2002 [23]. In the current study, 2 patterns of *pvdhfr* combination alleles, i.e., triple (81.8%) and double (18.2%) mutant alleles were found in 2005, and all isolates possessed the mutant 58R and 117N/T alleles. The most prevalent *pvdhfr* and *pvdhps* combination alleles were triple mutant *pvdhfr* 58R/61M/117N allele combined with single mutant *pvdhps* 553G allele (78.8%) (Table 2), suggesting increased level of SP resistant *P. vivax* isolates in the Philippines.

Three patterns of *pvdhfr* combination alleles were found in samples collected from Bangladesh, i.e., quadruple (32.3%), triple (54.8%, 58R/61M/117N), and double (12.9%) mutant alleles. No mutant *pvdhps* allele was detected, indicating that *P. vivax* isolates circulating in Bangladesh possessed high pyrimethamine resistance genotype, but not for the sulfadoxine in 2010.

The mutants *pvdhfr* 58R and 117N/T were shown to be the dominant alleles in Southeast Asia, i.e., Thailand [12,13,15,22], Vietnam and the Philippines [23], East Timor [24], Myanmar [25], and Indonesia [14]. The mutants *pvdhps* 383G and 553G alleles were observed at low frequencies in most geographic regions including East Timor [24], Korea [25], Iran [18,21], and Pakistan [17,21], with highest frequency in Thailand [17,21,27]. The diversity of mutations in *pvdhfr* and *pvdhps* suggest different intensity of selective pressure resulting from SP uses for treatment of *P. falciparum* in 3 countries in Asia. This drug should not therefore be used in these countries for treatment of *P. vivax* infection.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

REFERENCES

1. Mint Lekweiry K, Ould Mohamed Salem Boukhary A, Gaillard T, Wurtz N, Bogreau H, Hafid JE, Trape JF, Bouchiba H, Ould Ahmedou Salem MS, Pradines B, Rogier C, Basco LK, Briolant S. Molecular surveillance of drug-resistant *Plasmodium vivax* using *pvdhfr*, *pvdhps* and *pvmdr*1 markers in Nouakchott, Mauritania. J Antimicrob Chemother 2012; 67: 367-374.
2. Rahman MM, Ortega L, Rastogi RM, Thimasarn K. Antimalarial drug resistance. Regional Health Forum 2011; 15: 52-56.
3. Annual Epidemiological Surveillance Report 2012. Available from: http://www.who.int/malaria/publications/.../profile_phl_en.pdf. 2012.
4. Wijeyaratne PM, Valecha N, Joshi AB, Singh D, Pandey S. An inventory on malaria drug resistance in Bangladesh, Bhutan, India and Nepal. Environmental Health Project 2004. p 1-43.
5. Gregson A, Plowe CV. Mechanisms of resistance of malaria parasites to antifolates. Pharmacol Rev 2005; 57: 117-145.
6. Kaur S, Prajapati SK, Kalyanaraman K, Mohmmed A, Joshi H, Chauhan VS. *Plasmodium vivax* dihydrofolate reductase point mutations from the Indian subcontinent. Acta Trop 2006; 97: 174-180.
7. Mayxay M, Pukrittayakamee S, Newton PN, White NJ. Mixed-species malaria infections in humans. Trends Parasitol 2004; 20: 233-240.
8. Snounou G, White NJ. The co-existence of *Plasmodium*: sidelights from falciparum and vivax malaria in Thailand. Trends Parasitol 2004; 20: 333-339.
9. Zakeri S, Najafabadi ST, Zare A, Djadid ND. Detection of malaria parasites by nested PCR in south-eastern, Iran: evidence of highly mixed infections in Chahbahar district. Malar J 2002; 1: 2.
10. Mehlotra RK, Lorry K, Kastens W, Miller SM, Alpers MP, Bockarie M, Kazura JW, Zimmerman PA. Random distribution of mixed species malaria infections in Papua New Guinea. Am J Trop Med Hyg 2000; 62: 225-231.
11. Imwong M, Pukrittayakamee S, Rénia L, Letourneur F, Charlieu JP, Leartsakulpanich U, Looareesuwan S, White NJ, Snounou G. Novel point mutations in the dihydrofolate reductase gene of *Plasmodium vivax*: evidence for sequential selection by drug pressure. Antimicrob Agents Chemother 2003; 47: 1514-1521.
12. Thongdee P, Kuesap J, Rungsihirunrat K, Tippawangkosol P, Mungthin M, Na-Bangchang K. Distribution of dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps) mutant alleles in *Plasmodium vivax* isolates from Thailand. Acta Trop 2013; 128: 137-143.
13. Kuesap J, Rungsrihirunrat K, Thongdee P, Ruangweerayut R, Na-Bangchang K. Change in mutation patterns of *Plasmodium vivax* dihydrofolate reductase (*pvdhfr*) and dihydropteroate synthase (*pvdhps*) in *P. vivax* isolates from malaria endemic areas of Thailand. Mem Inst Oswaldo Cruz 2011; 106 (Suppl 1): 130-133.
14. Hastings MD, Porter KM, Maguire JD, Susanti I, Kania W, Bangs MJ, Sibley CH, Baird JK. Dihydrofolate reductase mutations in *Plasmodium vivax* from Indonesia and therapeutic response to sulfadoxine plus pyrimethamine. J Infect Dis 2004; 189: 744-750.
15. Ranjitkar S, Schousboe ML, Thomsen TT, Adhikari M, Kapel CM, Bygbjerg IC, Alifrangis M. Prevalence of molecular markers of anti-malarial drug resistance in *Plasmodium vivax* and *Plasmodium falciparum* in two districts of Nepal. Malar J 2011; 10: 75.
16. Imwong M, Pukrittakayamee S, Looareesuwan S, Pasvol G, Poirriez J, White NJ, Snounou G. Association of genetic mutations in *Plasmodium vivax dhfr* with resistance to sulfadoxine-pyrimethamine: geographical and clinical correlates. Antimicrob Agents Chemother 2001; 45: 3122-3127.
17. Zakeri S, Afsharpad M, Ghasemi F, Raeisi A, Safi N, Butt W, Atta H, Djadid ND. Molecular surveillance of *Plasmodium vivax dhfr* and *dhps* mutations in isolates from Afghanistan. Malar J 2010; 9: 75.
18. Afsharpad M, Zakeri S, Pirahmadi S, Djadid ND. Molecular assessment of *dhfr*/*dhps* mutations among *Plasmodium vivax* clinical isolates after introduction of sulfadoxine/pyrimethamine in combination with artesunate in Iran. Infect Genet Evol 2012; 12: 38-44.
19. Barnadas C, Kent D, Timinao L, Iga J, Gray LR, Siba P, Mueller I, Thomas PJ, Zimmerman PA. A new high-throughput method for simultaneous detection of drug resistance associated mutations in *Plasmodium vivax dhfr, dhps* and *mdr1* genes. Malar J 2011; 10; 282.
20. Barnadas C, Tichit M, Bouchier C, Ratsimbasoa A, Randrianasolo L, Raherinjafy R, Jahevitra M, Picot S, Ménard D. *Plasmodium vivax dhfr* and *dhps* mutations in isolates from Madagascar and therapeutic response to sulphadoxine-pyrimethamine. Malar J 2008; 7: 35.
21. Zakeri S, Motmaen SR, Afsharpad M, Djadid ND. Molecular characterization of antifolates resistance-associated genes (*dhfr* and *dhps*) in *Plasmodium vivax* isolates from the Middle East. Malar J 2009; 8: 20.
22. Rungsihirunrat K, Sibley CH, Mungthin M, Na-Bangchang K. Geographical distribution of amino acid mutations in *Plasmodium vivax* DHFR and DHPS from malaria endemic areas of Thailand. Am J Trop Med Hyg 2008; 78: 462-467.
23. Auliff A, Wilson DW, Russell B, Gao Q, Chen N, Anh LN, Maguire J, Bell D, O'Neil MT, Cheng Q. Amino acid mutations in *Plasmodium vivax* DHFR and DHPS from several geographical regions and susceptibility to antifolate drugs. Am J Trop Med Hyg 2006; 75: 617-621.
24. Almeidaa AD, do Rosáriob VE, Henriquesb G, Arezb AP, Cravob P. *Plasmodium vivax* in the democratic Republic of East Timor: parasite prevalence and antifolate resistance-associated mutations. Acta Trop 2010; 115: 288-292.
25. Lu F, Lim CS, Nam DH, Kim K, Lin K, Kim TS. Mutations in the antifolate resistance-associated genes dihydrofolate reductase

and dihydropteroate synthase in *Plasmodium vivax* isolates from malaria-endemic countries. Am J Trop Med Hyg 2010; 83: 474-479.
26. Zakeri S, Afsharpad M, Ghasemi F, Raeisi A, Kakar Q, Atta H, Djadid ND. *Plasmodium vivax*: prevalence of mutations associated with sulfadoxine-pyrimethamine resistance in *Plasmodium vivax* clinical isolates from Pakistan. Exp Parasitol 2011; 127: 167-172.
27. Imwong M, Pukrittayakamee S, Grüner AC, Rénia L, Letourneur F, Looareesuwan S, White NJ, Snounou G. Practical PCR genotyping protocols for *Plasmodium vivax* using *pvcs* and *pvmsp1*. Malar J 2005; 4: 20.