



The Brazilian Journal of INFECTIOUS DISEASES

www.elsevier.com/locate/bjid



Brief communication

Sequence analysis of genes associated with resistance to chloroquine and sulphadoxine pyrimethamine in *P. falciparum* and *P. vivax* isolates from the Bannu district of Pakistan

Lubna Khatoon^{a,*}, Frederick N. Baliraine^b, Salman A. Malik^a, Guiyun Yan^c

^a Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan

^b Department of Biology, LeTourneau University, Longview, TX, USA

^c College of Health Sciences, University of California, Irvine, CA, USA

ARTICLE INFO

Article history:

Received 11 July 2012

Accepted 4 February 2013

Available online 11 July 2013

Keywords:

Plasmodium vivax

Plasmodium falciparum

Sequence analysis

Pakistan

ABSTRACT

Plasmodium vivax and *Plasmodium falciparum* are becoming resistant to drugs including antifolates, sulphonamides and chloroquine. This study was focused at sequence analysis of resistant genes of these parasites against sulphadoxine–pyrimethamine and chloroquine, from Bannu, Pakistan. Known mutations were detected at codons 57, 58 and 117 of *pvdhfr* gene of *P. vivax*, while none of the isolates had any *pvdhps* mutation. Similarly *P. falciparum* isolates exhibited double 59R + 108N mutations in *pf dhfr*, and single 437G in *pf dhps* thus demonstrating the existence of triple mutant 59R + 108N + 437G haplotype in this region. The key chloroquine resistance mutation, 76T in *pf crt* was observed in 100% of the *P. falciparum* isolates, with haplotype SVMNT which is also associated with resistance to amodiaquine. Some novel mutations were also observed in *pvdhfr* and *pf dhfr* genes.

© 2013 Elsevier Editora Ltda. All rights reserved.

Plasmodium vivax and *Plasmodium falciparum* have been reported to develop resistance against drugs like sulphadoxine–pyrimethamine (SP) and chloroquine (CQ) in Pakistan.¹ The development of multidrug resistance by *P. falciparum* and chloroquine resistance by *P. vivax* and insecticide-resistant mosquitoes has led to major difficulties in controlling the spread of malaria. Previous studies have suggested that resistance to SP is attributed to the emergence of certain mutations that occur sequentially in dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*) genes of *Plasmodium*.^{2,3} Rigorous work has been

conducted to study the mutation structure of *dhfr* and *dhps* genes of *P. falciparum* but studies on *P. vivax dhfr* and *dhps* are limited to regions in Southeast Asia like Indonesia, Papua New Guinea, India and Sri Lanka.^{4,5}

While PCR-RFLP methodologies have been developed for the detection of known polymorphisms, sequence analysis enables the detection of previously unknown mutations that may be playing some role in drug resistance.² The sequencing approach is especially necessary for *P. vivax*, whose mechanisms of antimalarial drug resistance have not been clearly defined, although thought to be similar to those of *P. falciparum*.

* Corresponding author at: Quaid-i-Azam University, PO Box 1090, Islamabad 45320, Pakistan.

E-mail addresses: lubnakhatoonqau@gmail.com, lubnabch@hotmail.com (L. Khatoon).

1413-8670/\$ – see front matter © 2013 Elsevier Editora Ltda. All rights reserved.

http://dx.doi.org/10.1016/j.bjid.2013.02.005

Table 1 – Sequence analysis of portions of *Pvdhfr*, *Pvdhps*, *Pfdhfr*, *Pfdhps*, *Pfcr*, *PfmdrI* and *dhfr* genes of *P. vivax* and *P. falciparum* parasites encompassing the regions with known drug resistant target-codons. *P. vivax* and *P. falciparum* parasites were obtained from blood samples of malaria patients from Bannu, Pakistan.

Genes	Substitutions	Samples	Mutations	Accession ^a
<i>Pvdhfr</i>	149:A-T	PK43, PK47, PK49, PK54, PK64	Q49H	JN794518
	172:A-C	PK100	S58R	JN794521
	174:C-G	PK42, PK68, PK76	S58R	JN794517
	174:C-A	PK66	S58R	JN794520
	205:T-C, 207:T-C	PK60	Y69H	JN794519
	278,279:AG-CA	PK11, PK36, PK58, PK86, PK87	S93H ^b	JN794515
	350:G-A	PK25, PK30, PK34, PK42, PK43, PK46, PK47, PK49, PK54, PK57, PK60, PK64, PK66, PK68, PK79, PK100, PK102	S117N	JN794516
	<i>Pvdhps</i>	Wild type ^c		
<i>Pfdhfr</i>	175:T-C	PK1, PK4, PK6, PK7, PK8, PK10, PK19, PK53, PK55, PK63, PK71, PK74, PK85, PK101, PK105, PK106, PK108, PK109, PK110, PK111, PK112	C59R	JN794523
	323:G-A	PK1, PK4, PK6, PK7, PK8, PK10, PK19, PK53, PK55, PK63, PK71, PK74, PK85, PK101, PK105, PK106, PK108, PK109, PK110, PK111, PK112	S108N	
	493:G-A	PK1, PK4, PK5, PK6, PK7, PK8, PK10, PK19, PK41, PK53, PK55, PK63, PK74, PK85, PK101, PK109	G165R ^b	
<i>Pfdhps</i>	69:C-G	PK6, PK63, PK85, PK109, PK112	A437G	JN794524
<i>PfmdrIa</i>	230:A-T	PK53, PK72,	N86Y	JN794525
<i>PfmdrIb</i>	Wild ^c			JN794526
<i>Pfcr</i>	87:T-A95,96:TG-GA98:A-T	PK1, PK5, PK6, PK53, PK112	K76T	

^a Could not be submitted to GenBank due to short sequence size (≤ 200 bp).

^b Novel mutation.

^c Mutation observed in one or two isolates were ignored and hence regarded as wild type.

This study is an extension of our previous study⁶ where drug resistance has been studied through PCR-RFLP. The objective was to know the overall gene sequence of *P. falciparum* and *P. vivax* isolates from Bannu district in Pakistan; a region with high prevalence of malaria, but where molecular studies of malaria parasites are particularly scarce.

As described earlier,⁶ two samples from patients infected with *P. falciparum*, 69 carrying *P. vivax* and 26 having mix infection were sequenced to analyze the frequency of drug resistance mutations. Sequence analyses was conducted at the *Pfcr*, *Pfdhfr*, *Pfdhps*, *PfmdrI*, *Pvdhfr*, and *Pvdhps* genes using primers already published that allow to amplify fragments encompassing several codons in each gene.^{2,7-9} A total of 160 genes were cloned and sequenced consisting 10 *Pfcr*, 23 *Pfdhfr*, 22 *Pfdhps*, 20 *PfmdrI* spanning codon 86 and 10 *PfmdrI* spanning codons 1042 and 1246; 40 *Pvdhfr*, and 35 *Pvdhps*. Each fragment was cloned using the TA cloning kit (Invitrogen) according to manufacturer's instructions. Bioedit and CLC DNA online softwares were used to investigate the phylogenetic relationships among the fragments of the genes analyzed.

Table 1 summarizes the single nucleotide polymorphisms (SNPs) detected in the studied genes. 711 bp of the *pvdhfr* gene sequence spanning codons 13, 33, 57, 58, 61, 117 and 173 were compared with the sequence of gene with a *P. vivax* isolate collected from Pakistan (GenBank accession no. X98123.1). Novel mutations were confirmed after sequencing at least two clones for each fragment. The majority of single nucleotide polymorphisms (SNPs) led to synonymous changes, with few exceptions (Table 1). A change from A to T at position 149 resulted in the substitution of a Q for an H in 12.5% of the samples. Similarly, two adjacent SNPs at positions 77 and 78 resulted in the emergence of a novel mutant type S93H.

Similarly, we found a mutation of two nucleotides at positions 278 and 279 leading to S93H mutation in 16.5% of the *P. vivax* samples. However, the role of these mutations in conferring resistance against SP is still to be explored. Compared to the wild type reference, 17.5% of samples showed an insertion or deletion of an 18 bp fragment (ACACACGGTGGTGACAAT) which was not taken into consideration during nucleotide count). Phylogenetic tree in Fig. 1A shows that 7 clusters of *pvdhfr* were obtained. Sample PK11 is closely related to the wild type strain shown as *pvdhfr* in the figure. However, it has less similarity with other isolates. 705 bp region of *pvdhps* gene was sequenced and compared with wild type isolate from GenBank (accession no. AY186730.1). There was no change at codons 383 and 553.

Sequences of *Pfdhfr* gene spanning codons 16, 50, 51, 59, 108 and 164 were compared with GenBank sequence (accession no. J04643.1). Sequence homology among these isolates is represented by the neighbor joining phylogenetic tree in Fig. 1B. Sequence variation at *pfdhps* locus was determined by comparing sequence data of samples with wild type isolate from GenBank (accession no. Z30659.1). In 31.8% of the isolates, a SNP at position 69 led to the mutation A437G. A 145 bp region of the *pfcr* gene was compared with GenBank wild type isolate (accession no. AF030694.2) and it was found that all the samples carried SVMNT haplotype. The sequence data of the *pfmdrI* gene were compared with wild type sequence from GenBank (accession no. X56851.1). All samples carrying any non-synonymous mutation and a representative wild type sequences were submitted to the GenBank (Table 1).

This study was aimed at sequencing the entire genes involved in drug resistance to find out the involvement of any possible new mutations in conferring drug resistance. Though

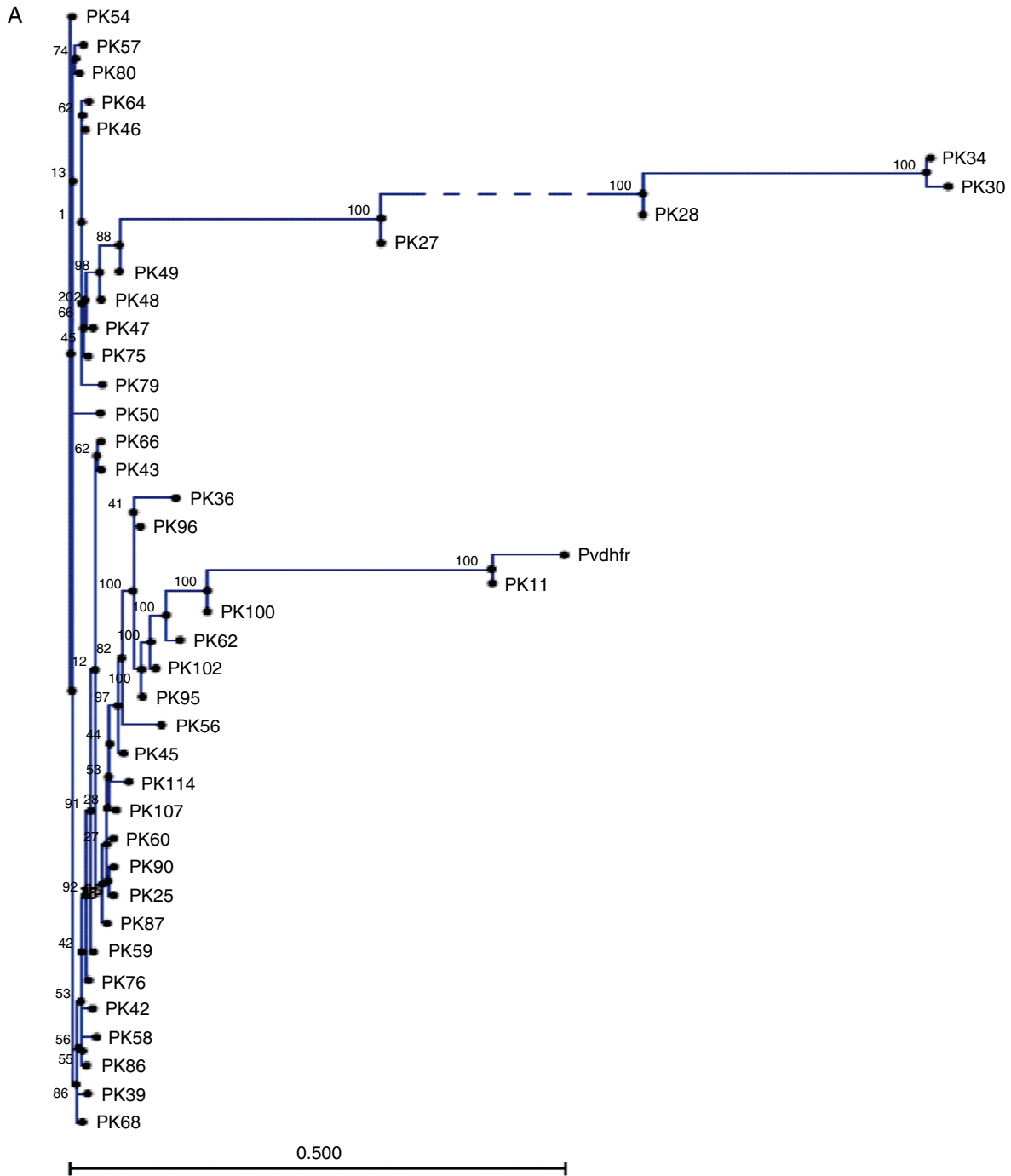


Fig. 1 – Phylogenetic representation of *pvdhfr* gene (A) and *pfdhfr* (B) along with wild type sequences from GenBank.

it has been found earlier that SP drug resistance is prevalent in Bannu and PCR-RFLP showed some known mutations,⁶ to have the genetic picture of the organisms it was important to sequence the entire genes. So our aim was to identify the level of polymorphism and possibly new mutations that need to be studied further in order to assess their implication on clinical efficacy of SP. SP is effective in patients having *P. vivax* infection with wild-type genotype or even a 58R-117N double mutant allele¹⁰ so other mutations are clearly involved. Sequence analysis showed polymorphism in this gene and some unique mutations like Q49H, Y69H and S93H have also

been found but their role in conferring drug resistance against SP is still to be elucidated.

No *pvdhfr* mutation was detected in isolates from our study area. Therefore, it can be said that Bannu is an area where SP pressure is low, similar to earlier findings.⁹

Mutations in *pfdhfr* gene are said to emerge in a stepwise manner⁴ with S108N emerging initially followed by N51I or C59R and lastly I64L mutant type. By comparison we observed mutations at codons 59 and 108 in all our isolates but no mutation was observed at codons 51. McCollum and colleagues while working with African isolates opposed this theory by

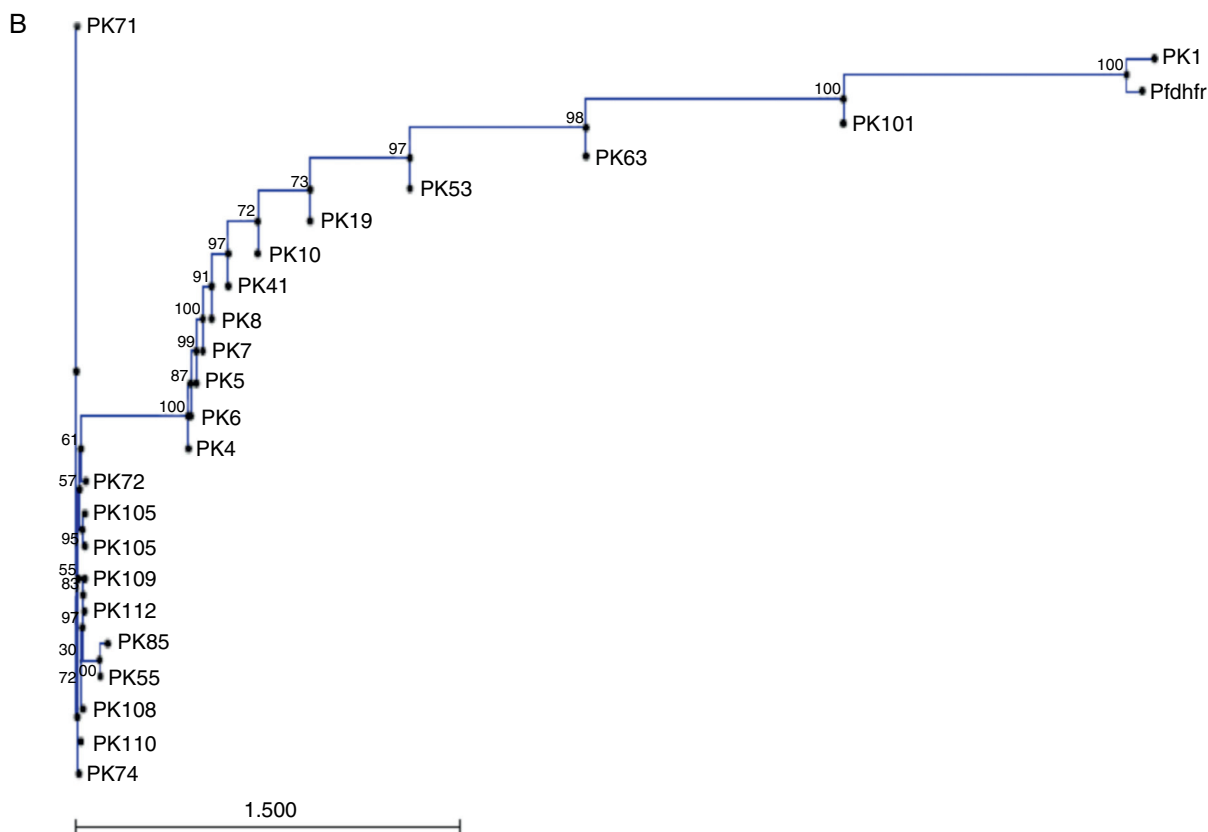


Fig. 1 – (Continuación).

finding 51I/108N/164L genotype.¹¹ We did not find A16V and S108T mutations that are thought to be responsible for resistance against the drug Cycloguanil which again support the findings of Garg and colleagues.¹² A non-synonymous mutation G165R was detected in *pfdhfr* gene which to our knowledge is a novel mutation.

The majority of isolates were found to contain wild-type *pfdhps* gene.

We detected haplotype 86Y-1042Y, in *Pfmdr1* gene unlike in West Africa where 86Y-86N-1246D haplotype occurred more commonly.¹³ Mayengue and colleagues while working with isolates from Gabonese patients found *Pfmdr1* 86Y and 184F mutations in their samples which are in accordance with our findings though we did not analyze mutations at codon 184.¹⁴

We found haplotype SVMNT in all our isolates which is also found in isolates from Papua New Guinea and South America. The SVMNT haplotype of *pfcr1* is also considered to be linked with chloroquine resistance, and, like the K76T mutation, it is common among Indian field isolates¹⁵ which is in accordance with our findings. CVIET haplotype is more prevalent in Asia and Africa while SVMNT is associated with Papua New Guinea and South American isolates¹⁶ while SVIET, CVIKT, CVIDT and CVTNT have been found in Southeast Asia.¹⁷ High level of mutation in *pfcr1* gene shows that *P. falciparum* has developed resistance against CQ. It could be due to the fact that areas like Pakistan where mixed infections by both *P. vivax* and *P. falciparum* exist and CQ is prescribed for treatment of *P. vivax*

malaria thus inadvertently putting *P. falciparum* under selective drug pressure which leads to emergence of CQ resistance, as observed in the Mekong Delta.¹⁸

In this study, sequence analysis of key genes associated with antimalarial drug resistance showed that *P. falciparum* and *P. vivax* in the Bannu district of Pakistan have not yet reached an alarming threshold against drugs like sulfadoxine-pyrimethamine unlike in many parts of the world but resistance seems to be on the rise. However, *P. falciparum* seem to have already acquired considerable resistance against CQ. Moreover, some novel mutations, whose roles in malaria drug resistance are unknown, were identified.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Higher Education Commission of Pakistan for financial support of this research work for PhD of Ms. Lubna Khatoun at Quaid-i-Azam University Islamabad Pakistan and University of California, Irvine, USA.

REFERENCES

1. Khan MA, Smego Jr RA, Razi ST, Beg MA. Emerging drug-resistance and guidelines for treatment of malaria. *J Coll Physicians Surg Pak*. 2004;14:319-24.
2. Imwong M, Pukrittayakamee S, Re'nia L, et al. Novel point mutations in the dihydrofolate reductase gene of *P. vivax*: evidence for sequential selection by drug pressure. *Antimicrob Agent Chemother*. 2003;47:1514-21.
3. Alker A, Mwapasa V, Purfield A, et al. Mutations Associated with sulfadoxine-pyrimethamine and chlorproguanil resistance in *P. falciparum* isolates from Blantyre, Malawi. *Antimicrob Agent Chemother*. 2005;49:3919-21.
4. Gregson A, Plowe CV. Mechanisms of resistance of malaria parasites to antifolates. *Pharmacol Rev*. 2005;57:117-45.
5. Hawkins VN, Joshi H, Rungsihirunrat K, Na-Bangchang K, Sibley CH. Antifolates can have a role in the treatment of *P. vivax*. *Trends Parasitol*. 2007;23:213-22.
6. Khatoun L, Baliraine FN, Bonizzoni M, Malik SA, Yan G. Prevalence of antimalarial drug resistance mutations in *P. vivax* and *P. falciparum* from a malaria-endemic area of Pakistan. *Am J Trop Med Hyg*. 2009;81:525-8.
7. Duraisingh MT, Jones P, Sambou I, Seidlein VL, Pinder M, Warhurst DC. The tyrosine-86 allele of the *pfmdr1* gene of *P. falciparum* is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. *Mol Biochem Parasitol*. 2000;108:13-23.
8. Lopes D, Rungsihirunrat K, Nogueira F, et al. Molecular characterisation of drug-resistant *P. falciparum* from Thailand. *Malar J*. 2002;1:12.
9. Imwong M, Pukrittayakamee S, Cheng Q, et al. Limited polymorphism in the dihydropteroate synthetase gene (*dhps*) of *P. vivax* isolates from Thailand. *Antimicrob Agent Chemother*. 2005;49:4393-5.
10. Hastings MD, Porter KM, Maguire JD, et al. Dihydrofolate reductase mutations in *P. vivax* from Indonesia and therapeutic response to sulfadoxine plus pyrimethamine. *J Infect Dis*. 2004;189:744-50.
11. McCollum AM, Poe AC, Hame M, et al. Antifolate resistance in *P. falciparum*: multiple origins and identification of novel *dhfr* alleles. *J Inf Dis*. 2006;194:189-97.
12. Garg S, Saxena V, Kanchan S, et al. Novel point mutations in sulfadoxine resistance genes of *P. falciparum* from India. *Act Trop*. 2009;110:75-9.
13. Dippmann AK, Bienzlem U, Harms G, Mockenhaupt FP. *Pfmdr1* mutations in imported African *P. falciparum* isolates. *Trans R Soc Trop Med Hyg*. 2008;102:1148-50.
14. Mayengue PI, Kalmbach Y, Issifou S, Kremsner PG, Ntoumi F. No variation in the prevalence of point mutations in the *Pfcr*t and *Pfmdr1* genes in isolates from Gabonese patients with uncomplicated or severe *P. falciparum* malaria. *Parasitol Res*. 2007;100:487-93.
15. Vathsala PG, Pramanik A, Dhanasekaran S, et al. Widespread occurrence of the *P. falciparum* chloroquine resistance transporter (*pfcr*t) gene haplotype SVMNT in *P. falciparum* malaria in India. *Am J Trop Med Hyg*. 2004;70:256-9.
16. Wootton JC, Feng X, Ferdig MT, et al. Genetic diversity and chloroquine selective sweeps in *P. falciparum*. *Nature*. 2002;418:320-3.
17. Nagesha HS, Casey GJ, Rieckmann KH, et al. New haplotypes of the *P. falciparum* chloroquine resistance transporter (*pfcr*t) gene among chloroquine-resistant parasite isolates. *Am J Trop Med Hyg*. 2003;68:398-402.
18. Congpuong K, Bangchang NK, Mungthin M, Bualombai P, Wernsdorfer WH. Molecular epidemiology of drug resistance markers of *P. falciparum* malaria in Thailand. *Trop Med Int Health*. 2005;10:717-22.