Malaria Journal

BioMed Central

Methodology

A new method for detection of *pfmdr1* mutations in *Plasmodium falciparum* DNA using real-time PCR

Anne Purfield[1], Amy Nelson[2], Anita Laoboonchai[3], Kanungnij Congpuong[4], Phillip McDaniel[5], R Scott Miller[3], Kathy Welch[5], Chansuda Wongsrichanalai[3,6] and Steven R Meshnick*[1,2]

Address: [1]Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC, U.S.A, [2]Department of Epidemiology, University of North Carolina School of Public Health, Chapel Hill, NC, U.S.A, [3]Department of Immunology, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand, [4]Bureau of Vector Diseases, Department of Disease Control, Ministry of Public Health, Thailand, [5]Kwai River Christian Hospital, Sangkhlaburi, Kanchanaburi, Thailand and [6]U.S. Naval Medical Research Unit, Jakarta, Indonesia

Email: Anne Purfield - purfield@med.unc.edu; Amy Nelson - apickard@email.unc.edu; Anita Laoboonchai - Anintita.Laoboonchai@AMEDD.ARMY.MIL; Kanungnij Congpuong - Robert.Miller.2@amedd.army.mil; Phillip McDaniel - Robert.Miller.2@amedd.army.mil; R Scott Miller - Robert.Miller.2@amedd.army.mil; Kathy Welch - Robert.Miller.2@amedd.army.mil; Chansuda Wongsrichanalai - chansuda@namru2.med.navy.mil; Steven R Meshnick* - meshnick@unc.edu

* Corresponding author

Published: 07 May 2004

Malaria Journal 2004, **3**:9

Received: 06 February 2004
Accepted: 07 May 2004

This article is available from: http://www.malariajournal.com/content/3/1/9

Abstract

Background: Surveillance for drug-resistant *Plasmodium falciparum* should be a component of malaria control programmes. Real-time PCR methods for the detection of parasite single-nucleotide polymorphisms (SNPs) and gene amplification could be useful survellance tools.

Methods: A real-time PCR assay has been developed that identifies single nucleotide polymorphisms (SNPs) at amino acids 86, 184, 1034 and 1042 in the *P. falciparum* multi-drug resistant (*pfmdr 1*) gene that may be associated with anti-malarial drug resistance.

Results: This assay has a sensitivity and specificity of 94% and 100% when compared to traditional PCR methods for genotyping. Only 54 of 68 (79%) paired pre- and post-culture DNA samples were concordant at all four loci.

Conclusion: Real-time PCR is a sensitive and specific method to detect SNP's in *pfmdr 1*. Genotypes of parasites after *in vitro* culture may not reflect that seen *in vivo*.

Background

The increasing prevalence of multi-drug resistant parasites threatens to impede efforts to control malaria world-wide [1]. Current *in vitro* and *in vivo* methods to monitor the emergence of drug resistance are difficult, costly and labor-intensive. Molecular methods could prove to be useful alternatives [2].

For *Plasmodium falciparum*, the *pfmdr 1* gene product, PGH1, is thought to play an integral role in the mechanism behind parasite resistance to multiple malarial drugs [3]. Both increased gene copy number and single-nucleotide polymorphisms (SNP's) have been associated in epidemiological studies with changes in sensitivity to chloroquine and mefloquine (reviewed in [1,4,5]). In two

studies from South-east Asia, both increased *pfmdr 1* gene copy number and SNPs have been associated with *in vitro* resistance to mefloquine [6,7].

In this manuscript, a real-time PCR assay has been developed for the detection of *pfmdr 1* SNPs. This assay was developed to be used in conjunction with a previously developed assay for *pfmdr 1* gene copy number [6] to assess molecular markers as predictors of mefloquine failure in a clinical study that took place in Sangkhlaburi, Thailand, in 2001–2. The results of the clinical study will be described in a subsequent publication.

Numerous studies have shown discrepancies between *in vitro* and *in vivo* tests for antimalarial drug resistance [8-11]. In order to understand why, *pfmdr 1* genotypes were determined directly from patient blood and from cultures derived from those patients and compared.

Methods

Patient samples

Subject recruitment. This study received ethical approval from the University of North Carolina IRB and the Thai Ministry of Public Health. Patients presenting with slide-confirmed falciparum malaria to the free Ministry of Public Health malaria clinic or the Kwai River Christian Hospital clinic in Sangklaburi, Thailand from during July 2001 – August 2002 were enrolled. Patients with vivax infections, history of anti-malarial drug use within the past two weeks, bleeding tendency (by self-reported history or based on medical records), or severe/complicated malaria requiring prompt medical management for life support were excluded. In total, 74 patients consented. Blood samples were taken, aliquoted, stored in liquid nitrogen, and transported to the Armed Forces Research Institute for the Medical Sciences (AFRIMS) in Bangkok. At AFRIMS, aliquots were thawed for *in vitro* culture. 59 of the 74 patient blood samples were successfully cultured as previously described [6,12]. Parasites were cultured for between 2 and 188 days (mean = 38.5; median = 23). DNA was extracted from patient blood ("pre-culture") and cultured parasites ("post-culture") using the QiaAmp DNA Blood Minikit Blood and Body Fluid Spin Protocol (Qiagen, Valencia, CA) and then shipped on dry ice to the University of North Carolina.

Standard PCR

DNA was PCR-amplified using a MJ Thermocycler (MJ Research, Waltham, MA) as previously described [6]. DNA sequencing was performed at the University of North Carolina Sequencing Core facility using a 3100 Genetic Analyser (Applied Biosystems, Foster City, CA). The sequencing reaction was done using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Applied Biosystems).

Real-time PCR

For real-time PCR, pre-culture DNA and post-culture DNA were genotyped using an Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems). This assay uses sequence-specific fluorescently-labelled "MGB™ " probes to distinguish SNPs [13-15]. Primers and probes (Table 1) were designed using Primer Express Software, Version 2.0 (Applied Biosystems). Primers were obtained from Qiagen. Fluorescent MGB™ probes were

Table 1: Primers and probes used in real-time PCR assay

Pfmdr1		Oligonucleotide (5'--> 3')	Tm**
86	F	TGTATGTGCTGTATTATCAGGAGGAAC	63.1
	R	AATTGTACTAAACCTATAGATACTAATGATAATATTATAGG	61.7
	Asn (wt*) probe	6FAM-ACCTAAAT**T**CATGTTCTTT-MGB-NFQ	66.5
	Tyr (mut**) probe	VIC-ACCTAAAT**A**CATGTTCTTT-MGB-NFQ	65.8
184	F	AAGATGGACAATTTCATGATAATAATCCT	59
	R	AATACATAAAGTCAAACGTGCATTTTTTA	57.6
	Tyr (wt) probe	6FAM-CTTTTTAGGTTTAT**A**TATTTGGT-MGB-NFQ	65.2
	Phe (mut) probe	VIC-CTTTTTAGGTTTAT**T**TATTTGGT-MGB-NFQ	65.9
1034	F	AAAAAGAAGAATTATTGTAAATGCAGCTT	57.6
	R	GGATCCAAACCAATAGGCAAAA	58.9
	Ser (wt) probe	6FAM-ATTC**A**GTCAAAGCGCT-MGB-NFQ	65.8
	Cys (mut) probe	VIC-ATTC**T**GTCAAAGCGCT-MGB-NFQ	66.7
1042	F	AAAAAGAAGAATTATTGTAAATGCAGCTT	57.6
	R	TTTCCAGCATAACTACCAGTAAATATAAAAG	60.7
	Asn (wt) probe	6FAM-CAATTATTTATT**A**ATAGTTTTGC-MGB-NFQ	65.3
	Asp (mut) probe	VIC-AATTATTTATT**G**ATAGTTTTGC-MGB-NFQ	64.8

* Wild type (Asn86, Tyr1 84, Serl034 or Asnl042) sequence ** Mutant (Tyr86, Phe184, Cysl034 or Aspl042) sequence *** Melting temperature estimated by Primer press v2.0 For probes, the reporter dye is covalently attached at the 5'end (6FAM or VIC) with a minor groove binder (MGB) and non-fluorescent quencher (NFQ) covalently attached at the 3' end.

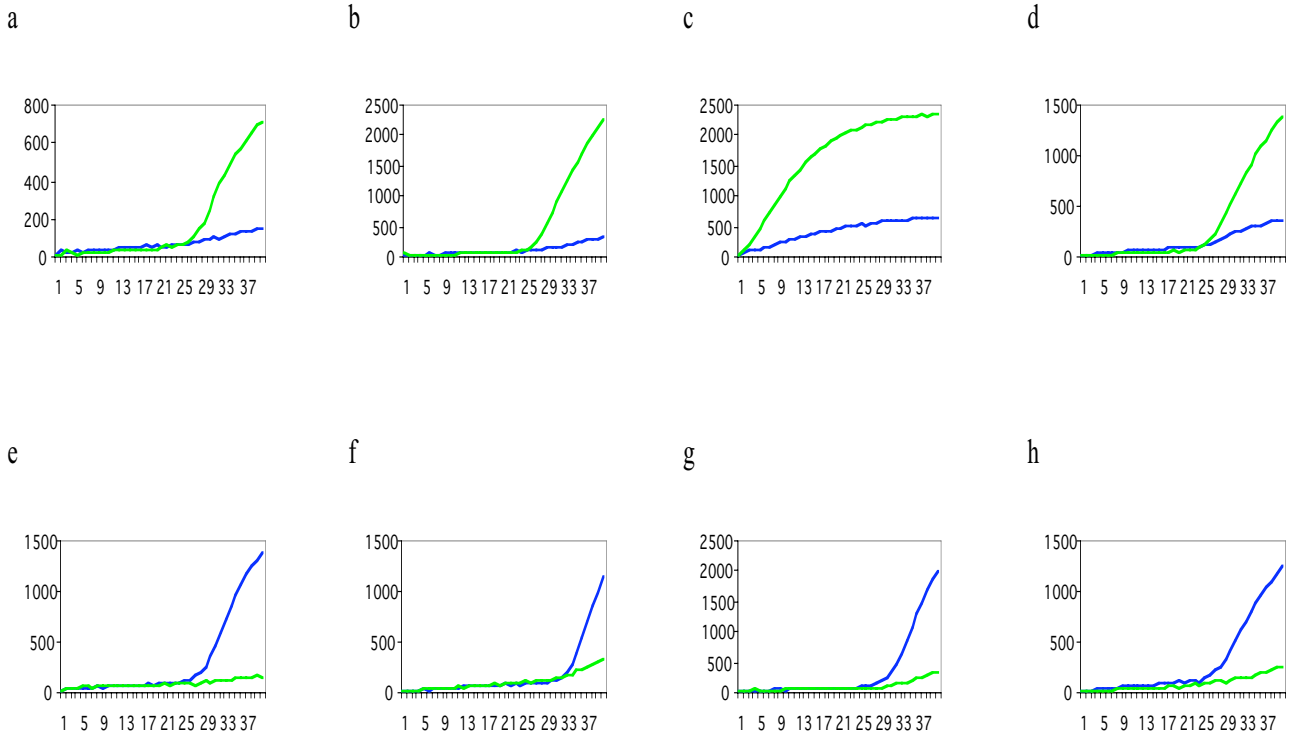

Figure 1
Fluorescent intensity (Rn) of the FAM reporter dye (blue line) compared to VIC reporter dye (green line) as PCR amplification cycles proceed (x axes). Fluorescent intensity is measured as Rn (y-axes), the absolute fluorescence of the reporter dye divided by that of the passive reference dye, Rox. An increase in FAM indicates a wild type population [86(e), 184(f), 1034(g), 1042(h)] while an increase in VIC indicates a mutant population [86(a), 184(b), 1034(c), 1042(d)]. Polyclonal infections may be identified by an increase in fluorescent intensity of both reporter dyes (not shown). Mutant 1034 DNA (c) was obtained from a previous study [6] because none of the samples in this study contained that mutation.

produced by Applied Biosystems and labelled with reporter dyes, 6FAM or VIC, at their 5' ends and non-fluorescent quencher molecules at their 3' ends. Primer and probe concentrations were optimized according to the TaqMan Universal PCR Master Mix technical bulletin (Applied Biosystems) using DNA obtained from *P. falciparum* strain 3D7 or from patient specimens. 300 nM of both forward and reverse primers were found to be optimal for all SNPs. Optimal probe concentration was determined to be 250 nM for all MGB probes used in this study. Using 10-fold serial dilutions of 3D7 genomic *P. falciparum* DNA, as few as 8 copies (0.001 ng) of genomic *P. falciparum* DNA could be detected.

Each real-time PCR amplification reaction contained both the wild type probe labelled with FAM, and the mutant probe labelled with VIC. Presence of either SNP could be imputed from the relative increase of fluorescence of the two fluorophores (Fig. 1). All reactions were done in duplicate.

Results

Parasite culture

Of the 74 patients enrolled, parasites were successfully cultured from 59. Following treatment, 20 of the 74 admission patients recrudesced. Parasites were successfully cultured from 13 of these 20 recrudescent patients.

Sensitivity and Ssecificity of Real-Time PCR

The sensitivity and specificity of SNP detection using real-time PCR were measured in 22 post-culture DNA samples using DNA sequencing as the gold standard (Table 2).

Table 2: Prevalence in monoclonal culture samples (n = 20) of mutations detected by traditional PCR and sequencing (gold standard) and real-time PCR, and sensitivity and specificity of realtime PCR assay.

	Prevalence		Real-time PCR	
	PCR	Real-time PCR	Sensitivity	Specificity
Tyr86	5%	5%	1/1 (100%)	19/19 (100%)
Phel84	65%		12/13 (92%)	7/7 (100%)
Cysl034	0%	0%	0/0 (--)	20/20 (100%)
Asp 1042	10%	10%	2/2 (100%)	18/18 (100%)
All mutations correct	--	--	15/16 (94%)	64/64 (100%)

Nineteen cultures were derived from blood of patients upon admission to the study. The other three cultures were obtained from who failed treatment. Two samples contained mixtures of SNPs at single positions based on DNA sequencing. Of the 20 monoclonal samples, 19 were concordant for all *pfmdr 1* SNPs when analysed using both methods. The discordant sample was not an artefact since both the real-time PCR and sequencing reactions gave identical results when repeated. The discordant sample contained a single discrepancy at position 184 (phenylalanine by sequencing and tyrosine by real-time PCR). Thus, the sensitivity of real-time PCR method to detect Tyr86 or Asp1042 SNPs was 100% and the sensitivity to detect Phel84 was 92%; there were no Cysl034 mutations detected. Real-time PCR was 100% specific for each mutation. The sensitivity and specificity of the assay to detect all four SNPs were 94% and 100%, respectively.

Two of the 22 samples were found to be mixed infections when sequenced. One sample contained a mixture of Tyrl84 and Phel84. The other sample contained a mixture of Asn86 and His86. The real-time PCR method accurately identified the Tyrl84/Phel84 mixed population. However, because the probes were designed to detect only Asn86 or Tyr86, His86 was not detected by real-time PCR.

Comparison of SNPs pre- and post-culture

In order to determine how the process of culturing parasites *in vitro* alters genotype, a comparison was made between the *pfmdr 1* genotypes of DNA extracted from pre-and post-culture parasites as measured by real-time PCR. Of the paired pre- and post-culture specimens, both members of 68 pairs were successfully amplified by real-time PCR. Fifty-eight of these pairs were obtained from admission patient blood while 10 pairs were derived from recrudescent patients.

Fifty-eight of the pre-culture genotypes were monoclonal, nine pre-culture samples had mixtures of alleles at single positions, and one had mixed SNPs at two positions (184 and 1042). Table 3 shows the number of isolates with specific combinations of *pfmdr 1* genotypes pre- and post-culture at each specific locus. Isolates with identical genotype at each locus before and after culture are enumerated in

Table 3: Genotype results of DNA from pre-culture and post-culture DNA by real-time PCR

Pre-culture genotype results	Post-culture genotype results									
Codon		86			184			1042		
	Amino Acid	Asn	Tyr	His	Tyr	Tyr & Phe	Phe	Asn	Asn & Asp	Asp
86	Asn	64	--	1						
	Tyr	1	2							
	His	--		--						
184	Tyr				27	1	2			
	Tyr & Phe				2	4	3			
	Phe				3	--	27			
1042	Asn							60	--	3
	Asn & Asp							1		1
	Asp							--	--	3

Data for codon 1034 was omitted because no mutant or polyclonal infections were detected in the 68 paired samples.

the shaded diagonal. Overall, fifty-four of 68 (79%) pre-culture DNA samples were completely concordant with post-culture DNA at all four polymorphic sites examined. While there was a wide variation in the length of time parasites were cultured before DNA was extracted, there was no correlation between genotype discordances and length of time in culture (data not shown). Five of the 14 discordant isolates exhibited single differences at position 184, and two were different at position 1042. Seven discrepant samples contained mixtures of alleles at one or more sites before and/or after culture. Six samples showed a mixture of alleles pre-culture and single alleles post-culture. This type of selection occurred at amino acid 184 for four isolates, and 1042 for two isolates. Two of these six also had changes at a second position, amino acid 86. Finally, one of the seven samples was monoclonal at aminoacid 184 pre-culture but polyclonal post-culture (and also exhibited a change from Asn to Asp at 1042).

Novel His86 mutation identified

In one DNA culture sample neither wild type nor mutant probes revealed amplification during real-time PCR. This post-culture sample was amplified by standard PCR methods and sequenced to genotype the position 86 region. Sequencing this sample revealed a His86.

Discussion

In this paper, a real-time PCR method is described which accurately ascertains parasite *pfmdr 1* genotype. This method has high sensitivity (94%) and specificity (100%) for detecting four *pfmdr 1* SNPs associated with drug resistance. There was a 21% discordance in the real-time PCR genotype results between DNA samples obtained directly from patient blood and DNA samples obtained from subsequent *in vitro* cultures.

Real-time PCR offers several advantages over standard PCR methods for genotyping DNA. First, because of the small volume the material, costs for real-time PCR are much lower than for standard PCR and are as little as $0.40 per reaction. Second, using real-time PCR, a single technician can perform and analyse hundreds of reactions per day, thus reducing the labor cost as well. Third, this method reduces the opportunity for post-PCR contamination. Once the sample is prepared with the reagents, amplification and analysis are completed in a closed-tube system. Finally, real-time PCR analysis of genotype is easier and requires less scientific expertise for analysis. Thus, the initial cost of a real-time PCR instrument (~$40,000) would be offset by savings in labor, quality assurance, and materials in labs analysing large numbers of samples.

In this paper, a novel His86 mutation was identified in two samples. To our knowledge, this mutation has never been identified before and may or may not be associated with drug resistance. Further studies on this mutation are needed.

A previous study utilized a novel real-time PCR technology to identify *pfmdr 1* SNPs in parasite DNA extracted from clinical blood samples [16]. Although the chemistry of the assay differed from the technique described here, both studies yielded similar results and concluded that real-time PCR may accurately detect $Asn^{86} \rightarrow Tyr^{86}$, $Tyr^{184} \rightarrow Phe^{184}$, $Ser^{1034} \rightarrow Cys^{1034}$ and $Asn^{1042} \rightarrow Asp^{1042}$ in *pfmdr 1*. In addition, de Monbrison, et al., used this method to genotype $Asp^{1246} \rightarrow Tyr^{1246}$. SNPs at 1246 were not studied here because they have not been found in South-east Asia [6,7].

Twenty-one percent of samples manifested differences in *pfmdr 1* genotype pre- and post-culture. Since most patients are probably infected with a mixture of strains, the genotypes observed post-culture reflects the results of the selection of a subset of strains by *in vitro* culture conditions. This could manifest itself as loss of strains observed pre-culture or the appearance of strains not observed pre-culture because they were present at levels below detection limits. A much greater change in genotype (74.5%) was observed in a previous study looking at variations in the polymorphic regions of MSP1 and MSP2 (merozoite surface protein 1 and 2) as well as GLURP (glutamate-rich protein) [17], perhaps because these genes are more variable.

In addition to SNP detection, our group has previously described a real-time PCR method to measure *pfmdr 1* gene amplification [6]. Thus it is now possible to assess both *pfmdr 1* gene copy number and SNPs using real-time PCR making it possible to carry out a complete assessment of *pfmdr 1* genetics in large cohorts.

Conclusions

Real-time PCR is a sensitive and specific method to detect *pfmdr 1* mutations and gene amplification. Because it is inexpensive and amenable to high-throughput, it could be a useful public health tool.

Acknowledgements

This work was supported by NIH grant R23 AI054590 and US Department of Defense-Global Emerging Infections Surveillance and Response Program (DoD-GEIS). We would like to acknowledge AFRIMS Malaria Field Team, Mark Fukuda, Paul Wilson, Charlotte Lanteri, Jesse Kwiek, Alisa Alker, the staff of Kwai River Christian Hospital and of the Vector Borne Diseases Control Unit #9 (Sangkhlaburi) for their assistance and support.

References

1. Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR: **Epidemiology of drug-resistant malaria.** *Lancet Infect Dis* 2002, **2:**209-218.
2. Plowe CV: **Monitoring antimalarial drug resistance: making the most of the tools at hand.** *J Exp Biol* 2003, **206:**3745-3752.

3. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF: **Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*.** *Nature* 2000, **403:**906-909.
4. Wernsdorfer WH, Noedl H: **Molecular markers for drug resistance in malaria: use in treatment, diagnosis and epidemiology.** *Curr Opin Infect Dis* 2003, **16:**553-558.
5. Talisuna AO, Bloland P, D'Alessandro U: **History, dynamics, and public health importance of malaria parasite resistance.** *Clin Microbiol Rev* 2004, **17:**235-254.
6. Pickard AL, Wongsrichanalai C, Purfield A, Kamwendo D, Emery K, Zalewski C, Kawamoto F, Miller RS, Meshnick SR: **Resistance to antimalarials in Southeast Asia and genetic polymorphisms in pfmdr1.** *Antimicrob Agents Chemother* 2003, **47:**2418-2423.
7. Price RN, Cassar C, Brockman A, Duraisingh M, van Vugt M, White NJ, Nosten F, Krishna S: **The pfmdr1 gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand.** *Antimicrob Agents Chemother* 1999, **43:**2943-2949.
8. Takechi M, Matsuo M, Ziba C, MacHeso A, Butao D, Zungu IL, Chakanika I, Bustos MD: **Therapeutic efficacy of sulphadoxine/pyrimethamine and susceptibility *in vitro* of *Plasmodium falciparum* isolates to sulphadoxine-pyremethamine and other antimalarial drugs in Malawian children.** *Trop Med Int Health* 2001, **6:**429-434.
9. Ringwald P, Basco LK: **Comparison of in vivo and in vitro tests of resistance in patients treated with chloroquine in Yaounde, Cameroon.** *Bull World Health Organ* 1999, **77:**34-43.
10. Segurado AA, di Santi SM, Shiroma M: ***In vivo* and *in vitro Plasmodium falciparum* resistance to chloroquine, amodiaquine and quinine in the Brazilian Amazon.** *Rev Inst Med Trop Sao Paulo* 1997, **39:**85-90.
11. Dua VK, Kar PK, Gupta NC, Kar I, Sharma VP: ***In vivo* and *in vitro* sensitivity of *Plasmodium falciparum* to chloroquine in Chennai (Tamil Nadu), India.** *Indian J Malariol* 1997, **34:**1-7.
12. Trager W, Jensen JB: **Human malaria parasites in continuous culture.** *Science* 1976, **193:**673-675.
13. Livak KJ: **Allelic discrimination using fluorogenic probes and the 5' nuclease assay.** *Genet Anal* 1999, **14:**143-149.
14. Afonina I, Zivarts M, Kutyavin I, Lukhtanov E, Gamper H, Meyer RB: **Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder.** *Nucleic Acids Res* 1997, **25:**2657-2660.
15. Holland PM, Abramson RD, Watson R, Gelfand DH: **Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of *Thermus aquaticus* DNA polymerase.** *Proc Natl Acad Sci U S A* 1991, **88:**7276-7280.
16. de Monbrison F, Raynaud D, Latour-Fondanaiche C, Staal A, Favre S, Kaiser K, Peyron F, Picot S: **Real-time PCR for chloroquine sensitivity assay and for pfmdr1-pfcrt single nucleotide polymorphisms in *Plasmodium falciparum*.** *J Microbiol Methods* 2003, **54:**391-401.
17. Viriyakosol S, Siripoon N, Zhu XP, Jarra W, Seugorn A, Brown KN, Snounou G: ***Plasmodium falciparum*: selective growth of subpopulations from field samples following *in vitro* culture, as detected by the polymerase chain reaction.** *Exp Parasitol* 1994, **79:**517-525.