Decreased in vitro susceptibility of Plasmodium falciparum Isolates to artesunate, mefloquine, chloroquine and quinine in Cambodia from 2001 to 2007

Running title: P. falciparum in vitro susceptibility to antimalarials drug in Cambodia

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Abbreviations:

1. AM: Combination of artesunate-mefloquine
2. AL: Combination of artemether-lumefantrine
3. ACPR: Adequate clinical and parasitological response
4. ACTs: Artemisinin derivatives based combination therapy
5. GMIC$_{50}$: Geometric mean IC$_{50}$
6. IC$_{50}$: 50% inhibitory concentration
7. LTF: Late treatment failure
8. MoH: Ministry of Health in Cambodia
9. MDR: Multidrug resistance
10. NMCP: Cambodian National Malaria Control Program
11. SNP: Single nucleotide polymorphism
12. SNPs: Single nucleotide polymorphisms
13. TES: Therapeutic efficacy studies
14. WHO: World Health Organization
Abstract

This study describes the results of antimalarial in vitro susceptibility assays and molecular polymorphisms of *Plasmodium falciparum* isolates from Cambodia. The samples were collected from patients enrolled in therapeutic efficacy studies (TES) conducted by the Cambodian National Malaria Control Program for the routine efficacy monitoring of artemisinin-based combination therapy or ACT (artesunate-mefloquine and artemether-lumefantrine combinations). The isolates (n=2041) were obtained from nine sentinel sites during 2001 – 2007. Among these, 1,588 were examined for their in vitro susceptibility to four antimalarials (artesunate, mefloquine, chloroquine and quinine) and 851 isolates were genotyped for single nucleotide polymorphisms (SNP).

The geometric means of the IC$_{50}$ (GMIC$_{50}$) were significantly higher for isolates from western than those from eastern Cambodia for the four drugs tested. Isolates from participants who failed artesunate-mefloquine therapy had significantly higher GMIC$_{50}$ than those from patients who were cured (p<0.001). In vitro correlation of artesunate with the other drugs was observed. The distribution of the SNP polymorphisms differed between eastern and western Cambodia, suggesting different genetic backgrounds of the parasite populations in these two parts of the country.

There was a significant increase in the GMIC$_{50}$ for the four tested drugs during 2006-2007 in eastern Cambodia. These results are worrisome as they may signal deterioration of the artesunate-mefloquine efficacy beyond the Cambodian-Thai border.

Keywords: artesunate, mefloquine, in vitro, *Plasmodium falciparum*, drug resistance, polymorphism, Cambodia


Introduction

Cambodia, especially the western border with Thailand, is known as the multi-drug resistant malaria hotspot. Pyrimethamine resistance was first reported on that border area in the early 1950s (27), followed by chloroquine resistance in the late 1950s, sulfadoxine-pyrimethamine (SP) resistance in the late 1960s, and mefloquine resistance in the late 1980s (31). Since 1986 (5), the Cambodian National Malaria Control Program (NMCP) has set up a program to monitor the efficacy of SP and mefloquine monotherapy, specifically in the provinces bordering Thailand, Vietnam and Laos. Between 1995 and 1999, failure rates based on 14-day follow-up periods were 7–30% for a low dose mefloquine (15 mg/kg), and 7–10% for the higher doses (20–25 mg/kg of mefloquine) in provinces in the northwest bordering Thailand, while the efficacy remained 100% elsewhere (Denis MB, personal communication). These findings were consistent with previous reports from Thailand (21). In 2000, studies of the clinical efficacy of artesunate-mefloquine (AM) (12 mg/kg artesunate and mefloquine 1000 mg were given over 3 days) were conducted, again in north-western Cambodia and confirmed 100% efficacy based on a 14-day follow-up (Denis MB, personal communication). NMCP decided to switch to AM combination therapy in the same year.

During an informal consultation on the monitoring of antimalarial drug efficacy in the Mekong Sub-Region organized by the World Health Organization (WHO) in October 2000 in Phnom Penh, it was agreed that each country should strengthen its capacity to monitor the efficacy of the first-line antimalarial drug. Monitoring should be conducted by therapeutic efficacy studies (TES) at selected sentinel sites on a regular basis, at least every other year for each site, with a 28-day follow-up. In 2001, the Center for Parasitology, Entomology and Malaria Control of Cambodia (CNM) conducted TES in two provinces, Sampovloun, in the
north-western part of the country near the Thai border, and Snoul, along the Vietnamese border.  
Both studies confirmed the high efficacy of AM with 96% and 100% adequate clinical and  
parasitological response (ACPR), respectively (7).  
In 2002, the failure rates of artesunate-mefloquine increased to 14.3% in the northwest  
part of the country along the Thai–Cambodian border (7). In vitro monitoring conducted on  
isolates from *Plasmodium falciparum* infected-patients during the years 2001 and 2002 showed  
significantly higher geometric mean IC$_{50}$s for mefloquine, chloroquine and quinine in the  
western provinces compared to eastern provinces (13). The molecular marker of chloroquine  
resistance is also known to be different in its geographic distribution in Cambodia; the *pfcrt*  
CVIETIF//ISS haplotype was detected in 92% of isolates from the West but only 11% of those  
from the East (14).  
Since 2001, regular monitoring of drug efficacy of artemisinin-based combination  
therapy (ACT) coupled with in vitro assessment and molecular marker assays has continued  
uninterruptedly. This article reports the temporal and geographic trends in the in vitro drug  
susceptibility assay results and prevalence of single nucleotide polymorphisms (SNPs) in isolates  
from infected patients enrolled in the TES during 2001 to 2007. These findings were also  
analysed in relation to the ACT clinical outcomes.  

Materials and Methods  
SITES AND SAMPLING  
Fresh clinical isolates of *P. falciparum* were collected from patients participating in  
therapeutic efficacy studies (TES or “in vivo studies”) of artesunate plus mefloquine (AM) and  
artemether plus lumefantrine (AL), conducted as a part of the routine antimalarial drug efficacy
monitoring of NMCP. Venous blood (5 mL) was collected in Venoject® tubes (Termo Europen.V.3001 Leuven, Belgium) containing EDTA and transported to Phnom Penh within 48h of collection at 4°C. Giemsa-stained thin blood smears were examined to determine parasite densities and to confirm *P. falciparum* mono-infection. When the parasitaemia ranged between 0.1% and 1%, drug sensitivity *in vitro* tests were performed directly. If parasite density exceeded 1%, samples were diluted with uninfected erythrocytes to obtain an initial parasitaemia between 0.5% and 1%. An aliquot of each isolate was frozen at –80°C for molecular analysis. If parasitaemia recurred, an additional blood sample was collected onto 3M Whatman filter paper and transported to the laboratory at room temperature, then kept at -20°C until DNA extraction.

**Therapeutic Efficacy Study (TES)**

The monitoring of ACT efficacy in Cambodia by TES has been carried out by CNM during 2001 – 2007 at 9 sentinel sites, namely, Sampovloun, Veal Veng and Pailin (western Cambodia), Preah Vihear and Anlong Veng (northern Cambodia), Ratanakiri and Snoul (eastern Cambodia), Chumkiri (southern Cambodia), and Oral (Kampong Speu province, central Cambodia). To simplify our analyses, we divided the study locations into the eastern region, including sites in the north and the east (Preah Vihear, Anlong Veng, Ratanakiri and Snoul), and the western region, including sites in the west, south, and central (Sampovloun, Veal Veng, Pailin, Chumkiri and Oral) (Figure 1).

These TES were performed according to the WHO 2003 protocol for low transmission areas and the clinical outcomes were previously published (6, 7). Briefly, all patients over 6 years of age and weight >16 kg presenting with fever (defined as axillary temperature ≥37.5°C or history of fever during 24 h prior to consultation) and a positive smear of *Plasmodium falciparum* mono-infection with parasite density of 1,000 – 150,000 parasites/µl, were included.
Informed consent was given by the participant or by a parent/guardian for children. Enrolled patients had to stay at the hospital/health center until completion of the 3-day AM or AL treatment regimen and blood smear became negative. They had to commit to a weekly follow-up for four weeks. Exclusion criteria were: one or more of the general danger signs or any sign of severe and complicated malaria, pregnancy, febrile diseases other than malaria, severe malnutrition, and known hypersensitivity or contraindication to the study drugs.

A total dose of 12 mg/kg artesunate and 25 mg/kg mefloquine were given over 3 days (AM group). The daily 4 mg/kg of artesunate were divided into two equal doses, one in the morning and one in the evening on day 0, and 4 mg/kg single dose on Day 1 and Day 2 with a maximum adult dose of 600 mg in total. Mefloquine (25 mg/kg) was also divided into two equal doses in the morning and in the evening on Day 0. The maximum dose of mefloquine was limited to 1,500 mg for adults as the side effects are common beyond this dose (7). For AL, participants received 20 mg/kg of artemether and 120 mg/kg of lumefantrine or a total adult dose of 24 tablets (Coartem®, Novartis, Switzerland) divided into two daily doses for three days. In the 2003 study, each dose of AL was provided with 250 ml milk and 5 pieces of coconut biscuit (6).

Participants were checked by blood smear for the presence of malaria parasites on study days 1, 2, 3, 7, 14, 21 and 28. If fever occurred at any time between the scheduled study days, a malaria smear was also done. Participants who did not return according to schedule on their own were actively sought for. The therapeutic response was classified as early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF) and adequate clinical and parasitological response (ACPR) according to WHO protocols (29). Late treatment failure
IN VITRO SUSCEPTIBILITY

Preparation of drugs and test plates

Quinine hydrochloride was obtained from Sigma (Steinheim, Germany). Mefloquine, chloroquine diphosphate and artesunate were obtained from RCC Ltd via Institut Pasteur in Paris, France. Stock solutions of chloroquine diphosphate and quinine were prepared in water (Biosedra, France) and stock solutions of mefloquine and artesunate were prepared in pure methanol. Subsequent two-fold serial dilutions were prepared in distilled water (Biosedra, France). The final concentrations ranged from 0.05 to 51.2nM for artesunate, 1 – 1024nM for mefloquine, 5 – 5120nM for chloroquine and 6.2 – 6400nM for quinine. Each concentration was used to coat two wells of a Falcon 96-well, flat-bottom plate (ATGC, France). Forty microliters of drug solutions prepared as described above were added to each well and plates were then air-dried in a laminar flow hood. The pre-dosed plates were stored at 4°C until use. Test plates were prepared weekly and used within two weeks after preparation. Their suitability for in vitro testing was regularly monitored using reference strains 3D7, maintained in continuous culture and presenting known responses to the various drugs tested.

Isotopic Assay

The drug sensitivity of the Cambodian isolates was assessed in vitro by the use of a classical isotopic (48 hr) test (8). Briefly, fresh blood samples were washed three times with RPMI 1640 medium (GibcoTM, Invitrogen Corporation, France) followed by centrifugation (800 × g, 10 min). The parasites were then tested directly without culture adaptation.
erythrocytes (1.5% hematocrit, 0.1 – 1% parasitaemia) were suspended in complete RPMI medium supplemented with 10% AB positive human serum inactivated for 30 min at 56°C (Biomedia, France) and buffered with 25 mM HEPES and 25 mM NaHCO₃. The mixture was transferred (200 µl per well) into the 96-well test plates that had been pre-coated with antimalarial agents. Each plate included two drug-free control wells and one control well without parasites. The culture plates were incubated for 48 hr at 37°C in a candle jar. [³H]-hypoxanthine (0.5 µCi/well; Amersham Biosciences, France) was used to assess parasite growth. Each isolate was tested in duplicate in microplates with serial dilutions of drugs. Drug response was quantified by measuring [³H]-hypoxanthine uptake in a Wallac MicroBeta Trilux counter (Perkin-Elmer, France). At the end of the incubation period, the plates were frozen at -20°C and thawed to lyse the cells. After collection on glass-fiber filter paper using a cell harvester, the amount of [³H]-hypoxanthine incorporated into parasite nucleic acids was determined. The results of the in vitro assay are expressed as the 50% inhibitory concentration (IC₅₀), defined as the concentration at which 50% of the incorporation of [³H]-hypoxanthine was inhibited by 50% of the drug-free control wells. Parasite growth was measured by using a log probit approximation to determine the IC₅₀ values.

**MOLECULAR ASSAYS**

**DNA extraction**

Parasite DNA was extracted from frozen blood aliquots (200µl) using High Pure PCR template preparation kit (Roche®, Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturers protocol. Blood stored on filter paper was extracted using QIAamp DNA Mini Kit (Qiagen, Giagen Gmbh, D-40724 Hilden) as previously described (11, 22).
Genetic polymorphism

Paired samples taken at enrolment and at recurrence of parasitaemia were used to distinguish between recrudescence and re-infection. The number of variants in three polymorphic genes (msp1, msp2 and glurp) was determined as described previously (11, 25). If the sample taken at recurrence contained either a subset of, or the same variants as the enrolment specimen, the infection was classified as recrudescence. If not, the infection was classified as a re-infection. The amplification of a single fragment at these three loci indicated that the parasite population was mono-infected (single genotype). Detection of two or more PCR bands, at one or more loci, indicated that the isolate contained multiple infections.

Nine SNPs of four genes: pfdhfr codons N51I, C59R, S108N/T, I164L; pfcrt codon K76T; pfmdr1 codons N86Y, Y184F, N1042D and pfatpase6 codon S769N were analysed using a microarray-based assay as described (4). Cut-off values were determined by an algorithm including absolute signal and relative (green/red) values of optical densities. Each signal was classified either as wild type or mutant (mixed was group as mutant) based on the expression intensities of the scanned image. Controls included previously sequenced samples and the 3D7 strain.

STATISTICAL ANALYSIS

All statistical analyses were performed using Stata SE 8 (Stata Corporation, College Station, Texas). Information on location, age, sex, parasitaemia and clinical outcome was collected in an anonymous database. The in vitro activity of the antimalarials was expressed as the geometric mean of the IC₅₀ for all isolates. Drug concentrations were transformed into logarithms. The Wilcoxon rank-sum (Mann-Whitney) test was used to determine whether the
observed differences in the in vitro responses to antimalarial drugs or treatment failure were significant. Linear regression analysis was used to assess the relationship between age, sex, the prevalence of parasite and \textit{pfmdr1} polymorphisms. The level of significance was adjusted using the Bonferroni correction. For all statistical tests, the significance level was set at $p=0.05$.

ETHICS

The study was approved by the National Ethics Committee for Health Research of the Cambodian Ministry of Health, the Institutional Review Board of the Naval Medical Research Unit No. 2 and the Technical Review Group of WHO/WPRO.

Results

A total of 2041 \textit{P. falciparum} isolates were collected during 2001 – 2007. Geographical location of the collection sites and the number of isolates collected per year per site are shown in Figure 1 and Table 1, respectively. Because of the discrepancy in the efficacy of AM between endemic areas in eastern and western Cambodia (7), further analyses were done based on the grouping of isolates into eastern and western Cambodia. Most of the data collected in 2001 and 2002, published earlier (13), are also included to serve as baseline values. This set of older data was re-analysed and standardized with the data from 2003 – 2007.

There were marked differences in the study subject demographics between the eastern and western sites. The median age was lower (17 vs 22, $p<0.001$, by Wilcoxon rank-sum (Mann-Whitney) test) and the proportion of male participants lower (0.6 vs 0.7, $p<0.001$, by $\chi^2$ test) in the east than in the west. However, the geometric mean parasitaemia was slightly higher for the west than the east (0.28\% vs 0.23\%, $p=0.001$, by Wilcoxon rank-sum (Mann-Whitney) test).
test). As expected, participants from eastern Cambodia responded to AM better than those from western Cambodia (ACPR = 98.7% vs 89.9%, p<.001, by $\chi^2$ test).

Out of the 2041 isolates, 1588 (78%) were examined for their in vitro sensitivity to four anti-malarial drugs; 274 isolates were excluded because of low parasitaemia (< 0.1%). An additional 179 isolates were not analysed because of delayed receipt of samples at the Institut Pasteur in Phnom Penh. 820 of the isolates analysed (52%) gave interpretable results for at least one of the drugs tested. That we did not attain a better success rate for the in vitro assays may be due to relatively low initial parasitaemias (<0.2%, N=778), suboptimal storage conditions, complications in transportation of samples, or possible presence of drug trace in the samples (although RBCs were washed three times before culture).

The overall geometric mean of IC$_{50}$ (GMIC$_{50}$) results are presented in Table 2. No significant correlation was found between IC$_{50}$ and sex, age or parasitaemia for any of the tested drugs. No attempt was made to test for the presence of antimalarial drugs in the blood samples. The GMIC$_{50}$ for all four tested drugs were significantly higher in the western than in the eastern provinces (p<0.001) (Table 3).

There were important differences in the GMIC$_{50}$ over time during the study period (2001-2007). The GMIC$_{50}$ for artesunate began to rise in the west in 2004, but remained low in the east until 2007, when it significantly increased (Table 4). Similarly the GMIC$_{50}$ for mefloquine began to increase in the west in 2004, but did not increase significantly in the east until 2007 (Table 5). The GMIC$_{50}$ for quinine and chloroquine have increased in both the western and eastern provinces from 2004, but the GMIC$_{50}$s for both drugs maintained their higher levels in the west (Tables 6, 7).
We next compared the GMIC$_{50}$ of the four tested drugs with clinical outcomes following AM treatment (Table 8). The results show that for all tested drugs, the GMIC$_{50}$ was significantly elevated in patients with LTF compared to those who were cured. No cut-off value was applied to the in vitro IC$_{50}$ level in order to determine its association with therapeutic failure.

There was a significantly positive correlation between the in vitro activity of artesunate and the other three drugs: mefloquine (Pearson $r = 0.338$, $n = 755$, $p < 0.001$), chloroquine (Pearson $r = 0.327$, $n = 770$, $p = 0.001$), and quinine (Pearson $r = 0.340$, $n = 738$, $p < 0.001$). The correlations were not significantly different when analysed separately for eastern and western Cambodia.

A total of 851 isolates from patients enrolled during 2003 – 2006 were randomly selected for a microarray analysis of gene polymorphism in four genes ($pf dhfr$, $pf c rt$, $pf md r1$ and $pf atpase6$). Interpretable results ranged from 52.4% for $pf md r1$ N1042D to 92.8% for $pf md r1$ N86Y. Table 9 summarizes the microarray results. Out of the 9 SNPs tested, 7 showed a mutation frequency greater than 5% (Figure 3). Of these, we observed 3 SNPs which showed a significant difference in their distributions between eastern and western Cambodia ($pf dhfr$ N51I, I164L and $pf md r1$ Y184F).

We did not find any SNPs showing a significant association either with year, parasitaemia, age, sex or the clinical outcome following AM therapy. $Pf md r1$ codon Y184F (N=114, $p<0.001$) and $pf dhfr$ codon I64L (N= 162, $p=0.02$) showed a significant association with elevated chloroquine GMIC$_{50}$ and $pf md r1$ codon Y184F was found to be associated with an increased level of artesunate GMIC$_{50}$ (N=113, $p=0.003$), but only for eastern Cambodia.
Discussion

There has been a major reduction in falciparum malaria cases in Cambodia for the past decade. The number of registered cases dropped from 130,000 in 2000 and to 60,000 in 2007. Malaria control was particularly successful along the Cambodian-Thai border and the malaria prevalence in that area is currently very low, an observation that is supported by the small number of isolates showing multiple infections. More than 82% of the isolates contained only one parasite population, with a single genotype at the *msp1*, *msp2* and *glurp* loci and the mean number of clones per isolate was only 1.18 (12).

Currently, there are several protocols for in vitro susceptibility assays of antimalarial drugs, with a concerted attempt to standardize the protocols. Here, the tests were performed following the recommendation of the WHO expert group for parasite culture, drug dilution and analysis (2). IC$_{50}$ results for the four drugs tested against a 3D7 cultured strain are in the range of acceptable values (Table 10). All susceptibility assays were done under the same conditions (same group of technicians, equipment and reagents) and the raw data were analysed together using the same software. Moreover, the in vitro tests were conducted at the same time, in isolates collected from western and eastern Cambodia, thus comparison of the data by time and place of sampling in this study is valid.

Our results show epidemiological differences between the eastern and western malaria endemic areas. For example, at the western sentinel sites there were significantly more young men (fewer children). These are explained by the fact that malaria in western Cambodia is more occupationally related. Adults tended to get exposed to malaria during their jungle trips while transmission in the villages is less and less common as malaria control is more successful in
wider areas. In eastern Cambodia, local transmission is more frequent in the villages therefore all age groups are affected.

In vitro IC\textsubscript{50} determination showed decreasing susceptibility for mefloquine, chloroquine, quinine and also artesunate during 2001 – 2007 in western Cambodia. These results are in support of the TES performed on the western border of Cambodia, which demonstrated the declining efficacy of AM therapy or suggested resistance to artesunate (7, 9, 15, 28).

We do not have a clear explanation why there was a dip in the geometric mean IC\textsubscript{50}s for every drug for the West in 2006. We do not believe this was a technical matter associated with the assays. It should be noted that sampling collection from the West in 2006 took place at one site only, i.e. in Chumkiri, located in the southwestern part of Cambodia and away from the Cambodian-Thai border area (Table 1). This was a new site as no samples had been taken from there prior to that year. Thus the results for 2006 may not be completely comparable to the previous years. Indeed the IC\textsubscript{50} values for the West in 2006 were lower than the prior year for all drugs. Due to the significance of Chumkiri as a new sentinel site of antimalarial drug resistance, we consider that it is important to including these findings. Interestingly, despite the lower geometric mean artesunate and mefloquine IC\textsubscript{50}s than other western sites, a high AM recrudescence rate (13.1% by Day 28) was observed in Chumkiri (19). In addition, among the isolates obtained from participants who suffered recrudescence, their mean IC\textsubscript{50}s for both mefloquine and artesunate were higher as compared to those who did not.

Previous studies showed that \textit{pfmdr1} copy number and mutation in codon 184 were both more prevalent in western than eastern Cambodia (23). Analysis of \textit{pfmdr1} copy number was also conducted in our laboratory and reported earlier (12). According to that report, the correlation between increased \textit{pfmdr1} copy number and increased mefloquine IC\textsubscript{50} was
statistically significant. However, we did not find a statistically significant correlation between increased \textit{pfmdr1} copy number and increased artesunate IC$_{50}$. Although the relationship between \textit{pfmdr1} copy number and artesunate in vitro sensitivity has been shown experimentally (24), field studies do not always demonstrate that relationship (9, 19) thus raising the suspicion that current in vitro assay methods may not be sensitive enough for detecting a change in artemisinin susceptibility levels in the field.

We confirm here the higher frequency of the \textit{pfmdr1} 184F mutant in western Cambodia. Recent studies have identified an association between \textit{pfmdr1} point mutations and decreased in vitro susceptibility to other antimalarial drugs including mefloquine, halofantrine, quinine and artemisinin (10, 20, 26, 32). The \textit{pfmdr1} 184 mutant was associated with increased IC$_{50}$ for mefloquine (16, 17). It is encouraging to further explore the significance of the \textit{pfmdr1} 184F mutation as a marker of mefloquine resistance.

The striking difference in the prevalence of mutant \textit{pfdhfr} 164 between eastern and western Cambodia (12.1\% versus 73.8\%, \(p<0.001\), by \(\chi^2\) test) is of interest. Although sulfadoxine-pyrimethamine is no longer of any therapeutic value in this malaria endemic area, a follow up to see if any changes occur over time with this marker may be epidemiologically important. The SNP data presented here may also serve as a baseline for the monitoring of some antimalarials to be deployed in the future.

The various SNPs found in this study neither correlated with the clinical outcome nor the in vitro IC$_{50}$ for any tested drug but their distinct geographic distribution suggests different genetic makeup of malaria parasites in the eastern and the western endemic areas. The different allelic distribution could be the result of selection and/or a drift acting on semi-isolated parasite sub-populations (1). Indeed, the areas are geographically separated by the natural barrier of the
Tonlé Sap channel, where rice fields are not suitable for the ecology of Anopheles (Figure 1).

However, we are aware that these preliminary conclusions need to be substantiated with a robust population genetics study.

The positive correlation detected among the antimalarial drugs including amino-alcohols, is consistent with previous reports (3, 13, 18) thus reflecting an alarming multi-drug resistant phenotype. It should be noted that the decreasing in vitro susceptibility observed in isolates from western Cambodia applied not only to drugs currently in operational use as the first line therapy against \textit{P. falciparum} (i.e. artesunate and mefloquine) but also chloroquine (only used for \textit{P. vivax}) and quinine (the second line drug against \textit{P. falciparum}).

In vitro drug susceptibility monitoring can be beneficial to a malaria control program in providing supportive data for the determination and prediction of the spread of resistance. In eastern Cambodia, the 2007 data showed a significant increase in the level of the GMIC$_{50}$ for the four drugs tested compared to the previous years. This trend is alarming as it suggests, particularly for mefloquine and artesunate, the deteriorating efficacy of these drugs that is no longer restricted to the Cambodian-Thai border but may be happening in other endemic areas in Cambodia as well, including the Cambodian-Vietnamese border.

\textbf{Authors' contributions}

PL contributed to the study design, performed experiments, data analysis and wrote the paper. CW contributed to the study design, data collection, discussion of the results and assisted in drafting the paper. PL, PC, SK, SC, RS and SN performed the in vitro assays. NK performed genetic polymorphism analyses. PY, SD, MBD coordinated and supervised the TES. BG, HPB, JG, WR and JYC contributed to the study design and writing of the manuscript. TF, OMP, PR...
and JLB initiated the study and contributed to the study design. FA contributed to the study
design, performed quality control of the data and helped to draft the paper. PR is a staff member
of the World Health Organization. The views expressed are the personal ones of the authors and
do not represent decisions, policies or views of the World Health Organization or the U.S. Navy.

Conflict of interest statement:

None declared.

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References


Figures

Figure 1 - Study sites of drug efficacy of falciparum malaria in Cambodia, 2001 - 2007

Figure 2 - Distribution of age and sex of the study subjects by geographic region (western and eastern Cambodia).

Figure 3 - Distribution of \( P. falciparum \) drug resistance SNPs in Cambodia

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Table 1 - Sample size of in vitro drug susceptibility tests by year and sentinel site

Table 2 - Overall in vitro susceptibility of \( P. falciparum \) isolates collected in the years 2001 - 2007

* Geometric mean 50% inhibitory concentration in vitro

Table 3 - Comparison of drug-specific GMIC\(_{50}\)s of \( P. falciparum \) isolates collected from eastern versus western Cambodia

† Geometric mean 50% inhibitory concentration in vitro with range

‡ Comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test

Table 4 - Comparison of artesunate IC\(_{50}\) values of \( P. falciparum \) isolates collected from eastern and western Cambodia (\( N = 820 \)) by year (2001 – 2007)

† Geometric mean 50% inhibitory concentration in vitro with range

‡ Comparison was tested by linear regression analysis

* \( p < 0.001 \), comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test between previous year in the same part of country (West or East)
Table 5 - Comparison of mefloquine IC$_{50}$ values of *P. falciparum* isolates collected from eastern and western Cambodia (N = 780) by year (2001 – 2007)

† Geometric mean 50% inhibitory concentration in vitro with range

‡ Comparison was tested by linear regression analysis

*p < 0.001, comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test between previous year in the same part of country (West or East)

**p ≤ 0.01, comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test between previous year in the same part of country (West or East).

Table 6 - Comparison of chloroquine IC$_{50}$ values of *P. falciparum* isolates collected from eastern and western Cambodia (N = 799) by year (2001 – 2007)

† Geometric mean 50% inhibitory concentration in vitro with range

‡ Comparison was tested by linear regression analysis

*p < 0.001, comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test between previous year in the same part of country (West or East)

**p ≤ 0.01, comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test between previous year in the same part of country (West or East).

Table 7 - Comparison of quinine IC$_{50}$ values of *P. falciparum* isolates collected from eastern and western Cambodia (N = 762) by year (2001 – 2007)

† Geometric mean 50% inhibitory concentration in vitro with range

‡ Comparison was tested by linear regression analysis

*p < 0.001, comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test between previous year in the same part of country (West or East)
**p≤0.01, comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test between previous year in the same part of country (West or East).

Table 8 - Comparison of GMIC_{50}s of *P. falciparum* isolates in the ACPR and LTF groups of the TES (28 day follow up) by drug

†Geometric mean 50% inhibitory concentration in vitro with range

‡Comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test

Table 9: Number and frequency of microarray-based SNP assays performed and mutants detected disaggregated by gene and specific point mutation

* A total of 851 isolates from patients enrolled were selected for microarray-based SNP test

Table 10 – GMIC_{50}s of the 3D7 *P. falciparum* clone tested for the in vitro susceptibility assay quality control at the Institut Pasteur in Cambodia during 2001 – 2007

† Geometric mean 50% inhibitory concentration in vitro
Table 1: Sample size of in vitro drug susceptibility tests by year and sentinel site

<table>
<thead>
<tr>
<th>Study area</th>
<th>Location</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anlong Veng</td>
<td>East</td>
<td>37</td>
<td>79</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>116</td>
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<tr>
<td>Chumkiri</td>
<td>West</td>
<td></td>
<td></td>
<td>97</td>
<td>55</td>
<td></td>
<td></td>
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<tr>
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<td>West</td>
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<td></td>
<td>97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>181</td>
</tr>
<tr>
<td>Pailin</td>
<td>West</td>
<td>81</td>
<td></td>
<td>86</td>
<td></td>
<td></td>
<td></td>
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<td>167</td>
</tr>
<tr>
<td>Preah Vihear</td>
<td>East</td>
<td>39</td>
<td>72</td>
<td></td>
<td>77</td>
<td>63</td>
<td></td>
<td></td>
<td>251</td>
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<tr>
<td>Ratanakiri</td>
<td>East</td>
<td>57</td>
<td>46</td>
<td>75</td>
<td></td>
<td>74</td>
<td></td>
<td></td>
<td>252</td>
</tr>
<tr>
<td>Sampovloun</td>
<td>West</td>
<td>75</td>
<td>101</td>
<td>138</td>
<td>45</td>
<td>41</td>
<td></td>
<td>400</td>
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</tr>
<tr>
<td>Snoul</td>
<td>East</td>
<td>118</td>
<td>42</td>
<td>64</td>
<td></td>
<td>77</td>
<td></td>
<td></td>
<td>301</td>
</tr>
<tr>
<td>Veal Veng</td>
<td>West</td>
<td></td>
<td>75</td>
<td>83</td>
<td></td>
<td>63</td>
<td></td>
<td></td>
<td>221</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>371</td>
<td>309</td>
<td>378</td>
<td>308</td>
<td>325</td>
<td>222</td>
<td>2041</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Overall in vitro susceptibility of *P. falciparum* isolates collected in the years 2001 – 2007

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>95% CI</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artesunate</td>
<td>0.94</td>
<td>0.89 - 1.01</td>
<td>0.05 - 11.1</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>23.57</td>
<td>22.05 - 25.19</td>
<td>1 - 181</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>141.74</td>
<td>135.10 - 148.70</td>
<td>7.16 - 814</td>
</tr>
<tr>
<td>Quinine</td>
<td>121</td>
<td>114.33 - 128.05</td>
<td>10.3 - 715.6</td>
</tr>
</tbody>
</table>

* Geometric mean 50% inhibitory concentration in vitro
Table 3: Comparison of drug-specific GMIC$_{50}$s of *P. falciparum* isolates collected from eastern versus western Cambodia

<table>
<thead>
<tr>
<th></th>
<th>Western Cambodia</th>
<th>Eastern Cambodia</th>
<th>p-value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>GMIC$_{50}$(range)† nM</td>
<td>n</td>
</tr>
<tr>
<td>Artesunate</td>
<td>495</td>
<td>1.2 (0.13 - 11.1)</td>
<td>325</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>468</td>
<td>27 (1 - 181)</td>
<td>312</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>469</td>
<td>170.1 (11.4 - 814)</td>
<td>330</td>
</tr>
<tr>
<td>Quinine</td>
<td>450</td>
<td>148.4 (15.1 - 715.6)</td>
<td>312</td>
</tr>
</tbody>
</table>

†Geometric mean 50% inhibitory concentration in vitro with range  
‡Comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test

Table 4 - Comparison of artesunate IC$_{50}$ values of *P. falciparum* isolates collected from eastern and western Cambodia (N = 820) by year (2001 – 2007)

<table>
<thead>
<tr>
<th></th>
<th>Western Cambodia</th>
<th>Eastern Cambodia</th>
<th>p-value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>GMIC$_{50}$(range)† nM</td>
<td>n</td>
</tr>
<tr>
<td>2001</td>
<td>74</td>
<td>1.1 (0.1 - 9.9 )</td>
<td>41</td>
</tr>
<tr>
<td>2002</td>
<td>102</td>
<td>1 (0.2 - 6.4)</td>
<td>55</td>
</tr>
<tr>
<td>2003</td>
<td>130</td>
<td>1.2 (0.2 - 11.1)</td>
<td>67</td>
</tr>
<tr>
<td>2004</td>
<td>48</td>
<td>1.8 (0.3 - 7.1)*</td>
<td>57</td>
</tr>
<tr>
<td>2005</td>
<td>51</td>
<td>1.6 (0.7 - 7.2)</td>
<td>Nd</td>
</tr>
<tr>
<td>2006</td>
<td>27</td>
<td>0.6 (0.1 - 1.6)*</td>
<td>77</td>
</tr>
<tr>
<td>2007</td>
<td>63</td>
<td>1.7 (0.4 - 6.7)*</td>
<td>28</td>
</tr>
</tbody>
</table>

†Geometric mean 50% inhibitory concentration in vitro with range  
‡Comparison was tested by linear regression analysis  
*p<0.001, comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test between previous year in the same part of country (West or East)
Table 5 - Comparison of mefloquine IC\textsubscript{50} values of \textit{P. falciparum} isolates collected from eastern and western Cambodia (N = 780) by year (2001 – 2007)

<table>
<thead>
<tr>
<th></th>
<th>Western Cambodia</th>
<th></th>
<th>Eastern Cambodia</th>
<th></th>
<th>p-value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>GMIC\textsubscript{50} (range)† nM</td>
<td>n</td>
<td>GMIC\textsubscript{50} (range)† nM</td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>71</td>
<td>23.6 (1.9 - 110.7)</td>
<td>41</td>
<td>17.3 (3.7 - 78.3)</td>
<td>0.023</td>
</tr>
<tr>
<td>2002</td>
<td>98</td>
<td>22.7 (4.5 - 110.1)</td>
<td>54</td>
<td>17.9 (6.3 - 88)</td>
<td>0.025</td>
</tr>
<tr>
<td>2003</td>
<td>120</td>
<td>12.2 (1 - 143.4)*</td>
<td>64</td>
<td>14.8 (1.9 - 63.7)</td>
<td>0.232</td>
</tr>
<tr>
<td>2004</td>
<td>43</td>
<td>57.2 (12.9 - 142.4)*</td>
<td>45</td>
<td>18.6 (1.5 - 99)**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2005</td>
<td>46</td>
<td>57.4 (28.4 - 139.5)</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>2006</td>
<td>27</td>
<td>42 (11.2 - 134.8)**</td>
<td>80</td>
<td>21.1 (2.2 - 131.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2007</td>
<td>63</td>
<td>54.1 (16.4 - 181)</td>
<td>28</td>
<td>36.6 (18.2 - 78.2)**</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

†Geometric mean 50% inhibitory concentration in vitro with range
‡Comparison was tested by linear regression analysis
*p<0.001, comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test between previous year in the same part of country (West or East)
**p≤0.01, comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test between previous year in the same part of country (West or East).

Table 6 - Comparison of chloroquine IC\textsubscript{50} values of \textit{P. falciparum} isolates collected from eastern and western Cambodia (N= 799) by year (2001 – 2007)

<table>
<thead>
<tr>
<th></th>
<th>Western Cambodia</th>
<th></th>
<th>Eastern Cambodia</th>
<th></th>
<th>p-value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>GMIC\textsubscript{50} (range)† nM</td>
<td>n</td>
<td>GMIC\textsubscript{50} (range)† nM</td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>73</td>
<td>131 (18 - 592)</td>
<td>41</td>
<td>79.2 (7.2 - 595)</td>
<td>0.002</td>
</tr>
<tr>
<td>2002</td>
<td>97</td>
<td>136.9 (11.4 - 650.6)</td>
<td>61</td>
<td>96.9 (11.4 - 674)</td>
<td>0.002</td>
</tr>
<tr>
<td>2003</td>
<td>123</td>
<td>167 (24.6 - 645.4)**</td>
<td>67</td>
<td>120.7 (13 - 414.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2004</td>
<td>39</td>
<td>236.5 (82 - 523.4)*</td>
<td>54</td>
<td>108.2 (20 - 441)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2005</td>
<td>49</td>
<td>216.4 (81.9 - 697.7)</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>2006</td>
<td>27</td>
<td>180.7 (83.7 - 422.2)</td>
<td>79</td>
<td>106.4 (15.9 - 362.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2007</td>
<td>61</td>
<td>221.2 (98.1 - 814)</td>
<td>28</td>
<td>199.9 (21.2 - 409.1)*</td>
<td>0.396</td>
</tr>
</tbody>
</table>

†Geometric mean 50% inhibitory concentration in vitro with range
Comparison was tested by linear regression analysis

*p<0.001, comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test between previous year in the same part of country (West or East)

**p≤0.01, comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test between previous year in the same part of country (West or East).

### Table 7 - Comparison of quinine IC$_{50}$ values of \textit{P. falciparum} isolates collected from eastern and western Cambodia (N = 762) by year (2001 - 2007)

<table>
<thead>
<tr>
<th>Year</th>
<th>Western Cambodia</th>
<th>Eastern Cambodia</th>
<th>p-value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>GMIC$_{50}$ (range)</td>
<td>n</td>
</tr>
<tr>
<td>2001</td>
<td>69</td>
<td>164.6 (28.8 - 583.5)</td>
<td>45</td>
</tr>
<tr>
<td>2002</td>
<td>94</td>
<td>93.6 (20.5 - 464.8)</td>
<td>50</td>
</tr>
<tr>
<td>2003</td>
<td>120</td>
<td>103.2 (15.1 - 538)</td>
<td>66</td>
</tr>
<tr>
<td>2004</td>
<td>40</td>
<td>226.6 (49.3 - 530.1)*</td>
<td>46</td>
</tr>
<tr>
<td>2005</td>
<td>45</td>
<td>230.3 (102.9 - 557.3)</td>
<td>Nd</td>
</tr>
<tr>
<td>2006</td>
<td>25</td>
<td>163.8 (58.5 - 496.9)**</td>
<td>77</td>
</tr>
<tr>
<td>2007</td>
<td>57</td>
<td>301.8 (104.1 - 715.6)*</td>
<td>28</td>
</tr>
</tbody>
</table>

†Geometric mean 50% inhibitory concentration in vitro with range

‡Comparison was tested by linear regression analysis

*Comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test between previous year in the same part of country (West or East)

**p≤0.01, comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test between previous year in the same part of country (West or East).
Table 8 - Comparison of GMIC_{50}s of *P. falciparum* isolates in the ACPR and LTF groups of the TES (28 day follow up) by drug

<table>
<thead>
<tr>
<th></th>
<th>ACPR n</th>
<th>GMIC_{50} (range)</th>
<th>LTF n</th>
<th>GMIC_{50} (range)</th>
<th>p-value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artesunate</td>
<td>558</td>
<td>0.83 (0.05-8)</td>
<td>37</td>
<td>1.72 (0.4-7.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>530</td>
<td>23.85 (1-181)</td>
<td>35</td>
<td>50.57 (6.8-142.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>551</td>
<td>139.15 (7.16-814)</td>
<td>36</td>
<td>179.43 (46.2-650.6)</td>
<td>0.0192</td>
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<tr>
<td>Quinine</td>
<td>516</td>
<td>113.03 (10.3-715.6)</td>
<td>36</td>
<td>201.14 (53.8-698.4)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

†Geometric mean 50% inhibitory concentration in vitro with range

‡Comparison was tested by Wilcoxon rank-sum (Mann-Whitney) test

Table 9: Number and frequency of microarray-based SNP assays performed and mutants detected disaggregated by gene and specific point mutation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Codon</th>
<th>Number of analysis (%)</th>
<th>Number of mutation (%) frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dhfr</td>
<td>N51I</td>
<td>774 (91%)</td>
<td>688 (88.9%)</td>
</tr>
<tr>
<td></td>
<td>C59R</td>
<td>754 (88.6%)</td>
<td>731 (96.9%)</td>
</tr>
<tr>
<td></td>
<td>S108N/T</td>
<td>580 (68.2%)</td>
<td>571 (98.5%)</td>
</tr>
<tr>
<td></td>
<td>I164L</td>
<td>644 (75.7%)</td>
<td>307 (47.7%)</td>
</tr>
<tr>
<td>Pfcr1</td>
<td>K76T</td>
<td>768 (90.2%)</td>
<td>764 (99.5%)</td>
</tr>
<tr>
<td>Pfmdr1</td>
<td>N86Y</td>
<td>790 (92.8%)</td>
<td>5 (0.6%)</td>
</tr>
<tr>
<td></td>
<td>Y184F</td>
<td>639 (75.1%)</td>
<td>352 (55.1%)</td>
</tr>
<tr>
<td></td>
<td>N1042D</td>
<td>446 (52.6%)</td>
<td>38 (8.5%)</td>
</tr>
<tr>
<td>Pfatpase 6</td>
<td>S769N</td>
<td>547 (64.3%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

* A total of 851 isolates from patients enrolled were selected for microarray-based SNP test
Table 10 - GMIC$_{50}$s of the 3D7 *P. falciparum* clone tested for the in vitro susceptibility assay quality control at the Institut Pasteur in Cambodia during 2001 – 2007

<table>
<thead>
<tr>
<th></th>
<th>GMIC$_{50}$ (nM) $^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$</td>
</tr>
<tr>
<td>Artesunate</td>
<td>38</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>37</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>35</td>
</tr>
<tr>
<td>Quinine</td>
<td>33</td>
</tr>
</tbody>
</table>

$^\dagger$ Geometric mean 50% inhibitory concentration in vitro