

Review Article

Plasmodium falciparum Resistance to Artemisinin Derivatives and Piperaquine: A Major Challenge for Malaria Elimination in Cambodia

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Abstract. Artemisinin-based combination therapies (ACTs) are the cornerstone of current strategies for fighting malaria. Over the last decade, ACTs have played a major role in decreasing malaria burden. However, this progress is being jeopardized by the emergence of artemisinin-resistant *Plasmodium falciparum* parasites. Artemisinin resistance was first detected in western Cambodia in 2008 and has since been observed in neighboring countries in southeast Asia. The problem of antimalarial drug resistance has recently worsened in Cambodia, with reports of parasites resistant to piperaquine, the latest generation of partner drug used in combination with dihydroartemisinin, leading to worrying rates of clinical treatment failure. The monitoring and the comprehension of both types of resistance are crucial to prevent the spread of multidrug-resistant parasites outside southeast Asia, and particularly to Africa, where the public health consequences would be catastrophic. To this end, new tools are required for studying the biological and molecular mechanisms underlying resistance to antimalarial drugs and for monitoring the geographic distribution of the resistant parasites. In this review, we detail the major advances in our understanding of resistance to artemisinin and piperaquine and define the challenges that the malaria community will have to face in the coming years.

BACKGROUND

In 2016, malaria remains a major human parasitic disease worldwide, affecting almost 3.2 billion people in 108 countries.¹ *Plasmodium falciparum*, the most lethal human malarial pathogen, is responsible annually for 214 million cases and 438,000 deaths.¹ Over the last decade, the malaria mortality rate has decreased considerably (by 48% worldwide), mostly due to the reinforcement of malaria control efforts and increases in the financial resources provided by both public and private international organizations. The prevention of malaria transmission, and the early detection and prompt treatment of malaria cases are currently the cornerstones of malaria control and elimination strategies. Malaria prevention is based on vector control measures (e.g., use of nets impregnated with long-lasting insecticide and the spraying of insecticides indoors) to limit contact between humans and vectors, thereby decreasing malaria transmission and morbidity. The management of suspected cases of malaria seen at health facilities or in the community is based on the early biological detection of malaria parasites by microscopy or rapid diagnostic tests, followed by effective and prompt treatment of the confirmed cases. These approaches limit the duration of the disease and prevent severe complications and death. Despite intensive efforts to develop effective vaccines and the promising results reported by vaccine trials (e.g., RTS,S/AS01 vaccine),² it will be several years before these new preventive weapons can be used in clinical practice.

Over the last century, all the antimalarial drugs deployed worldwide have inevitably led to the selection and spread of drug-resistant *P. falciparum* parasites, particularly in southeast Asia (Figure 1).^{3–13} In the 1950 and 1960s, the World Health Organization (WHO) Global Malaria Eradication Program was based on the massive deployment of chloroquine, along with

vector control measures (dichlorodiphenyltrichloroethane [DDT]). This program was initially highly successful, but the progress made was rapidly compromised by the emergence and spread of chloroquine-resistant parasites and DDT-resistant vectors. Later, in the 1970s and 1980s, a similar pattern was observed for alternative antimalarial drugs, such as sulfadoxine–pyrimethamine, which was used in areas in which chloroquine resistance had developed, leading to the selection of parasites resistant to antifolate agents. At the end of the 1990s,^{14,15} the global health situation, particularly in sub-Saharan Africa, was critical, with more than three million deaths annually due to malaria.^{16,17} In their attempts to control this alarming situation, stakeholders began to take an interest in derivatives of artemisinin, a sesquiterpene lactone discovered in 1972 by Chinese scientists during a program of research involving the screening of compounds from plants (Military code name: Project 523). Artemisinin derivatives, which are extracted from *Artemisia annua*, have been used to treat acute malaria in traditional Chinese medicine for more than 2,000 years. Novartis developed and patented the first artemisinin-based combination therapy (ACT), consisting of a combination of artemether and lumefantrine. The massive deployment of this new treatment in South Africa in 2000–2001 along with vector control measures was highly successful, decreasing the number of deaths from malaria by 87.5% in less than 1 year.¹⁸ In 2001, the WHO recommended the use of ACT as a first-line treatment of uncomplicated malaria in areas in which the parasites were resistant to monotherapy with other drugs, such as mefloquine.¹⁹ This recommendation was extended to the entire world in 2006, and the WHO banned the use of artemisinin in monotherapy to prevent the selection of artemisinin-resistant parasites. In 2012, five different ACTs, including dihydroartemisinin–piperaquine (DHA–PPQ), artesunate–mefloquine (AS–MQ), artemether–lumefantrine, artesunate–sulfadoxine–pyrimethamine, and artesunate–amodiaquine, were deployed in 79 countries with endemic malaria. At present, successful malaria control and, to a greater extent, malaria elimination rely strongly on effective antimalarial treatments. As no alternative to artemisinin derivatives are ready for deployment before 2020, emergence of resistance to artemisinin derivatives

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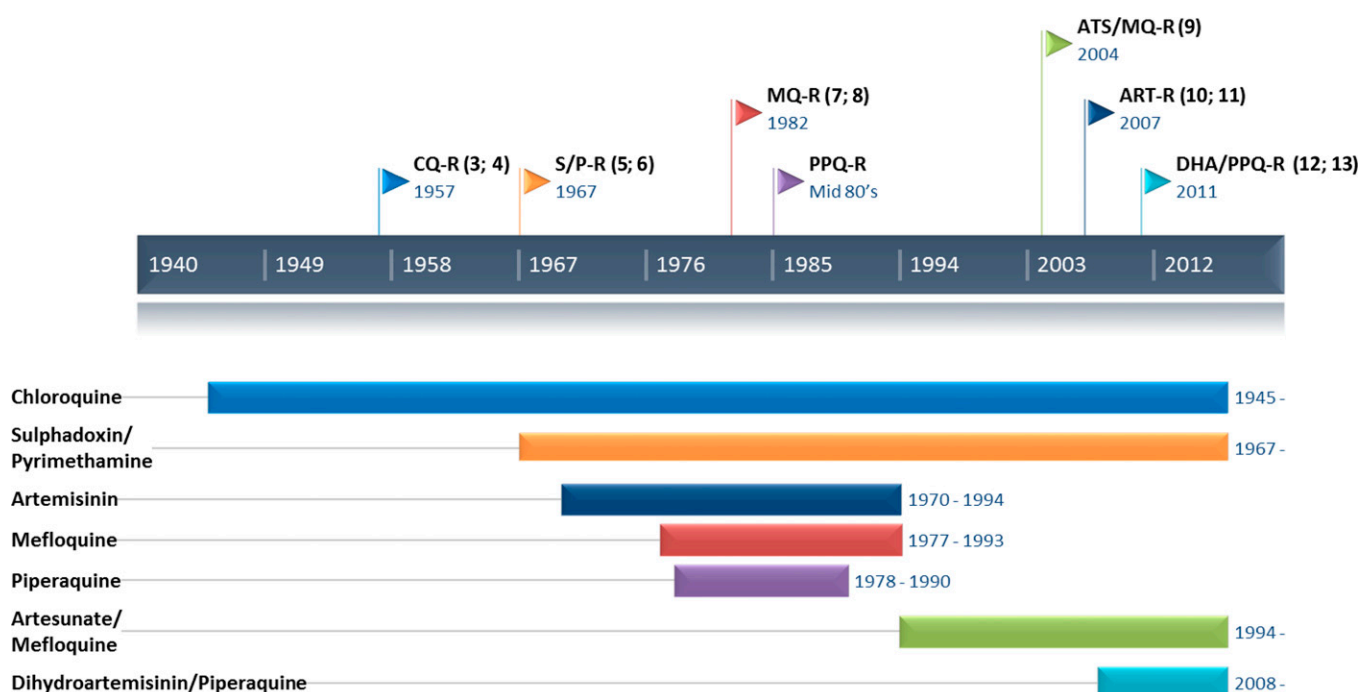


FIGURE 1. Timeline between the introduction of the main antimalarial drugs and the first case of emergence of resistance.^{3-5,7-13}

and partner drugs represents a major threat to malaria control and elimination efforts worldwide.

RESISTANCE TO ARTEMISININ DERIVATIVES: PAST AND PRESENT RESEARCH CHALLENGES

Clinical phenotype. Partial resistance to artemisinin in *P. falciparum* was first reported in 2008, in Battambang Province in western Cambodia.¹⁰ It was subsequently confirmed in 2009, in Pailin Province, the well-known epicenter of antimalarial multidrug resistance.¹¹ Artemisinin resistance has since been reported elsewhere in western Cambodia, western Thailand, southern Myanmar (Burma), and southern Vietnam,²⁰⁻²³ and in China.²⁴

Artemisinin resistance is defined clinically as delayed parasite clearance²⁵⁻²⁷ and a half-life for parasite clearance ≥ 5 hours has been associated with artemisinin resistance in southeast Asia.²⁷ In high-transmission settings, such as sub-Saharan Africa, where the partial immunity of adults plays a significant role in parasite clearance, the threshold need to be defined.²⁸ Currently, this approach is considered to be the gold standard, but suffers from several drawbacks: first, parasite clearance studies are both logistically and financially demanding, requiring the screening of thousands of febrile individuals to identify the small number of patients who will be hospitalized for several days; second, parasite clearance rates depend on many factors other than the intrinsic drug resistance of the parasites, relating to drug administration, immunity, and pharmacodynamics. Alternative in vivo approaches have been proposed. For instance, Beshir and others²⁹ assessed the association between parasite clearance following ACT and transmissibility in mosquitoes by membrane-feeding assays. They found that Kenyan children treated with a standard 3-day ACT course had commonly a persistent residual parasitemia

(assessed by quantitative polymerase chain reaction [qPCR]), associated with longer duration of gametocyte carriage and a higher likelihood of infecting mosquitoes. However, even being innovative and elegant, this approach is logistically demanding and not adapted for high-scale application.

Before 2013, the principal alternative approach for studies of artemisinin resistance in *P. falciparum* was the determination, in classical assays, of IC₅₀ values (the concentration inhibiting parasite growth by 50%) for DHA. However, all in vitro/vivo studies, except one,¹⁰ have reported no significant correlation²⁰ between clinical outcome (half-life for parasite clearance) and DHA IC₅₀ values. One potential reason for this observation was later put forward³⁰: the parasites in these assays were exposed to very low concentrations of DHA (≤ 64 nM) for 48–72 hours, whereas parasites in vivo are exposed to higher concentrations of drugs for only 1–2 hours.

Rethinking in vitro phenotype. A novel assay to overcome this problem was developed in 2012–2013. This assay was based on the exposure of culture-adapted or fresh isolates of *P. falciparum* parasites to the physiological conditions encountered in humans: *P. falciparum* parasites were exposed to 700 nM DHA (the active metabolite of all artemisinins) for 6 hours, reflecting typical doses and exposure times for ACTs. This assay (named ring-stage survival assay [RSA]) successfully detected significant differences in susceptibility in vitro between parasites originating from areas in which clinical resistance to artemisinin had and had not been observed.³⁰ In vitro artemisinin susceptibility was found to be stage dependent and strongly linked to the decrease in susceptibility of early ring stages. This further explains the lack of correlation between clinical outcome and DHA IC₅₀ values, during which the parasites are exposed to the drug throughout their cycle. These parasite stages were capable of surviving short periods of drug exposure by entering a state of developmental arrest

(dormancy).^{30–32} This observation was consistent with the findings of previous transcriptome studies showing that artemisinin-resistant parasites had a particularly long ring stage and lower metabolic activities³² and were also consistent with those of modeling studies suggesting a significant decrease in the efficacy of artemisinin derivatives against ring-stage parasites in western Cambodia as early as 2007–2008.³³ Survival rates in the RSA were also strongly correlated with clinical phenotype (half-life for parasite clearance).^{31,34}

A molecular marker. The molecular signatures associated with resistance to artemisinins were then investigated by sequencing the exome of an African strain (F32-Tanzania) that became resistant to artemisinin after repeated exposure to increasing concentrations of the drug over a period of 5 years (F32-ART).³⁵ The exome of F32-ART was compared with that of its sibling clone (F32-TEM) cultured under the same conditions but without the drug. A detailed analysis of the differences between the two exomes identified eight nonsynonymous single nucleotide polymorphisms (SNPs) in seven genes. Analysis of the genomes of three intermediate strains (after 22, 40, or 56 cycles of exposure) and of 49 strains isolated in Cambodia made it possible to determine the chronological order in which these mutations appeared. This analysis revealed a strong association between mutations in the propeller domain of the *Kelch* gene located on chromosome 13 (K13, PF3D7_1343700) and in vitro susceptibility to artemisinin in the RSA. Previous genome-wide association studies performed by two independent groups identified this region as associated with delayed parasite clearance half-lives.^{36,37} A study of the temporal (2001–2012) and spatial distribution of K13-mutant parasites showed that they had progressively spread in zones classified as “resistant” (western Cambodia) but not in areas in which artemisinin resistance had never been observed (eastern Cambodia). The detection of parasites with *K13* mutations in patients with slow rates of parasite clearance confirmed that this molecular marker was a major determinant of resistance to artemisinin derivatives.³⁸ Definitive evidence was later obtained in site-specific genome editing experiments using zinc-finger nucleases³⁹ or the CRISPR-Cas9 system.⁴⁰ This series of experiments showed that the replacement of a wild-type K13 allele in parasites susceptible to artemisinin by different K13 mutant alleles (Y493H, C580Y, M476I, R539T, I543T) conferred artemisinin resistance (survival rates assessed using the RSA increased from ≤ 1 –2% to 29% after the insertion of *K13* mutations), whereas the removal of *K13* mutations in isolates from Cambodia had the opposite effect (survival rates decreased from 13% to 49% to 0.3% to 2.4% after the removal of *K13* mutations).

This discovery provided unprecedented opportunities for improving strategies for the monitoring of artemisinin resistance. Several molecular epidemiology studies, mostly in southeast Asia,^{27,38,41–44} China,^{45–48} Indian subcontinent^{49,50} and Africa,^{27,51–58} investigated the frequency of *K13* mutant alleles, their spatial distribution and, in some cases, their association with clinical artemisinin resistance (day 3 positivity rate or parasite clearance half-life). Only 13 independent *K13* mutations of about 100 tested were found to be associated with clinical resistance, with evidence of the independent emergence of the same mutation in different areas. Only four of these mutations, all from Asia (C580Y, R539T, I543T, and Y493H), have been validated in vitro. A worldwide map of *P. falciparum* K13-propeller polymorphism was recently published from a study involving 41 partner institutions and

14,037 samples collected from 163 sites (in 59 countries in which malaria is endemic), providing a global overview of the distribution of artemisinin resistance and fundamental data for drug policy makers and future surveillance activities.⁵⁹ The main findings of the KARMA study (K13 Artemisinin Resistance Multicenter Assessment) suggest that artemisinin resistance remains confined to southeast Asia (including Cambodia, PDR of Laos, Thailand, Vietnam, and Myanmar) and China. In these two regions, the proportions of parasites with *K13* mutations were estimated at 51.8% (95% CI: 48.5–55.3%) and 25.6% (95% CI: 19.3–33.3%), respectively. Two independent foci of emergence were identified—one in Cambodia–Vietnam–PDR of Laos and the other in eastern Thailand–Myanmar–China—with no evidence of parasite flow between the two foci, consistent with other reports that showed that most *K13* mutations emerged independently from the same foci in southeast Asia, with the exception of mutations C580Y and Y493H, which probably emerged in Cambodia and subsequently spread to Lao PDR and Vietnam.⁴² Conversely, no K13 mutant alleles were found in South America, Oceania, the Philippines, and some of the countries in Central Asia. In Africa, the frequency of K13 mutant alleles (< 1.5%) was low. None African K13 mutant alleles were associated with artemisinin resistance and none of the Asian mutants known to be associated with resistance to artemisinin (C580Y, R539T, I543T, and Y493H) were found among the 9,542 African samples tested. The most frequent mutation in Africa, the A578S mutation (3.8%), which was previously detected in India, Bangladesh, and Thailand, was susceptible in the in vitro RSA (0–3 hours) and had no effect on positivity rate on day 3. Thus, not all nonsynonymous K13 mutants are associated with artemisinin resistance. The effective surveillance of artemisinin resistance must therefore be based on a combination of molecular data (genotyping to detect K13 mutants and haplotyping of the neighboring loci to assess the spread of mutant parasites carrying the same *K13* mutation) with clinical and in vitro studies (to validate the association of the mutations detected with artemisinin resistance). It is also worth noting that the lack of association between ACT treatment failures and K13 polymorphisms in Africa⁶⁰ could be a sign of K13-independent mechanisms, which need to be investigated. There is also a possibility that a particular genetic backbone is mandatory to increase the level of resistance conferred by *K13* mutations, and this specific genetic background is now only found in Asian parasite populations.⁶¹

Insight into resistance mechanisms. Despite the immense progress of recent years, the mechanisms involved in *P. falciparum* resistance to artemisinin derivatives remain poorly understood. Such an understanding is essential for the definition of new effective strategies to prevent the spread of resistant strains, and for the development of new detection tools and new drugs targeting these mechanisms. The protein encoded by the *K13* gene has a BTB/POZ domain (PDB 4YY8) and a C-terminal helical Kelch domain. The mutations associated with artemisinin resistance affect one of the β sheets of the Kelch domain (Y493H in blade 2, R539T and I543T in blade 3, and C580Y in blade 4). Such domains are frequently involved in protein–protein interactions and the validated mutations may alter the overall structure of the protein, substantially modifying the interactions between K13 and its potential partner proteins. Mbengue and others recently provided new insight into the mechanisms involved in K13-mediated artemisinin

resistance.⁶² They demonstrated that the presence of the *C580Y* mutation decreased polyubiquitination of the *P. falciparum* phosphatidylinositol-3-kinase (PfPI3K, which is strongly inhibited by artemisinins) by modifying its binding to K13, thereby limiting PfPI3K proteolysis and increasing production of the phosphatidylinositol-3-phosphate (PI3P). PI3P levels were found to be predictive of artemisinin resistance in both clinically and genetically modified *P. falciparum* strains, with high PI3P levels associated with an increase in artemisinin resistance in the absence of *K13* mutation.

Moreover, the K13 protein displays similarity to the human KEAP1 protein involved in the oxidative stress response mediated by the transcription factor Nrf2.^{63–67} Mutations of the *KEAP1* gene impairing the binding of the encoded protein to Nrf2 have been found in human cancers resistant to chemotherapy. This resistance is thought to be due to the diffusion of Nrf2 in the nucleus and the activation of several genes involved in the antioxidant responses (AREs), promoting cell survival.⁶⁸ A similar mechanism may account for the resistance of *P. falciparum* to artemisinin. It is therefore important to screen for proteins interacting with K13, to identify the cellular pathways involving K13 and to understand how these pathways are modified by *K13* mutations.

Perspectives. Major advances have been made since the detection of the first case of artemisinin resistance in 2008, and the identification of the *K13* mutation as a major determinant of artemisinin resistance has made it possible to conduct large-scale molecular monitoring programs with simple, inexpensive techniques (e.g., extraction of DNA from finger-prick blood samples on filter paper and its use for the detection of mutations by PCR and sequencing). Such programs can be used to monitor resistance in real time, making it possible to adapt antimalarial treatments according to the epidemiological situation in a given area. The timely implementation of such initiatives should make it possible to avert public health disasters like the spread of chloroquine-resistant parasites in Africa in the 1980s. Studies of the *K13* gene may also shed light on the cellular and molecular mechanisms underlying resistance to artemisinin derivatives, thereby making it possible to limit the spread of this resistance phenotype through the development of appropriate new drugs. However, as artemisinin derivatives are systematically administered with a partner drug, it is important to understand the mechanisms involved in the emergence of parasites resistant to partner drugs, particularly in areas of artemisinin resistance, in which the clearance of parasites from the bloodstream of patients depends primarily on the efficacy of the partner drug.

RESISTANCE TO PPQ: RESEARCH CHALLENGES

For decades, the Thai–Cambodian border has been the epicenter of multidrug-resistant *P. falciparum* malaria emergence. This region saw successively the demise of chloroquine (1960s), sulfadoxine–pyrimethamine (1970s), and MQ (1990s) treatments.¹⁴ MQ, initially introduced in the 1990s, was replaced, in 2000, by AS–MQ in Cambodia.⁶⁹ The clinical efficacy of this combination decreased rapidly, from 90.1% in 2001 to 79.4% in 2004,⁹ probably due to the high proportion of MQ-resistant parasites (high MQ IC₅₀ values and amplified *Pfmdr-1* gene copy).^{70–72} AS–MQ was replaced by DHA–PPQ^{73,74} in 2008, initially in western Cambodia (Pailin Province) and then throughout the entire country in 2010, as the

first-line drug of choice for uncomplicated malaria.⁷⁵ Compelling data, presented in Table 1 and Figure 2, clearly illustrate that the decrease of the ACT efficacy is always emerging in western Cambodia before to spread eastward. It is worth to note that AS–MQ regained efficacy promptly after it was replaced by DHA–PPQ (e.g., in Battambang, the reported efficacy of AS–MQ in 2004 was only 79.3% but reaches 100% in 2010, 1 year only after the implementation of DHA–PPQ as a first-line treatment in this area), suggesting that a switch from DHA–PPQ to AS–MQ could be a temporary solution in areas where DHA–PPQ is now failing. Clinical studies assessing artesunate–pyronaridine (ATS–PYRO) efficacy conducted in Cambodia were disappointing: indeed, ATS–PYRO did not meet the WHO efficacy criteria for first-line treatment of *P. falciparum* malaria in western Cambodia (i.e., >90% PCR-adjusted adequate clinical and parasitological response at day 42).⁷⁶ This was largely unexpected, given the high efficacy of this ACT in other Asian countries and in Africa.^{77–81} The reasons of this disparity remain unclear, but cross-resistance between pyronaridine and other antimalarial drugs like PPQ should be investigated.

DHA–PPQ is failing in Cambodia. The most recent clinical studies conducted in Cambodia between 2012 and 2015 have reported an alarming increase in the proportion of late clinical treatment failure following a standard 3-day course of DHA–PPQ. Treatment failure rates increased from 25% in 2010 to 46% in 2014^{20,82–85} in western Cambodia, suggesting that DHA–PPQ is becoming ineffective within only a few years of its nationwide deployment. The high treatment failure rates suggest that parasites with reduced susceptibility to both artemisinin and PPQ are now prevalent in western/northern Cambodia. Clinical failures of DHA–PPQ treatment have been linked to the emergence of PPQ resistance, but no strong correlation has been found between clinical outcome (recrudescence 2–4 weeks after the initial treatment) and susceptibility to PPQ in vitro, as assessed by the determination of IC₅₀ for pretreatment (day 0) isolates.⁸³ PPQ resistance remains poorly characterized and there is currently no robust evidence of parasite-dependent resistance to this drug (reliable in vitro phenotype and/or validated genetic molecular marker).

In vitro detection of PPQ resistance: Piperaquine Survival Assay. Several in vitro studies, conducted in Cambodia, have reported an increase over time in the geometric mean IC₅₀ of PPQ in western Cambodia or a significantly higher geometric mean IC₅₀ of PPQ for isolates from patients with recrudescence infections than for those from cured patients.²⁰ However, no direct association between a high IC₅₀ or 90% inhibitory concentration (IC₉₀) of PPQ for isolates before treatment and the failure of DHA–PPQ treatment has ever been demonstrated. In all published studies, the range of IC₅₀ values for PPQ in patients with recrudescence infections overlapped substantially with those in cured patients, suggesting that the classical in vitro test is not sufficiently reliable for the identification of PPQ-resistant parasites and would therefore probably detect the elimination, in a given area, of the parasites most sensitive to PPQ.^{20,84} It has been recently shown that the poor performance of the standard isotopic assays for detecting PPQ resistance was associated with a relatively high frequency of non-interpretable curves in assays on PPQ-resistant isolates. Most of the isolates collected from patients with recrudescence infection had non-interpretable IC₅₀ curves, whereas all yielded conventional response curves for the other drugs tested.

TABLE 1

Detailed data of the therapeutic efficacy studies performed in different sites in Cambodia between 2001 and 2015 to evaluate the efficacy of 3-day standard course of ACTs (AS–MQ, DHA–PPQ, ATS–PYRO, and A–L)

Study sites (province)	Start year	End year	Follow-up (days)	Antimalarial drug regimen	ACPR (%)	Reference
Battambang	2001	2001	28	AS–MQ	96.0	9
	2003	2003	28	AS–MQ	92.3	9
	2005	2005	42	AS–MQ	94.1	#
	2007	2007	42	AS–MQ	95.4	#
	2012	2013	42	DHA–PPQ	67.5	82
Kampong Speu	2014	2014	42	ATS–PYRO	100.0	#
	2003	2003	28	AS–MQ	96.7	9
	2010	2010	42	AS–MQ	88.9	93
Kampong Thom	2012	2013	42	DHA–PPQ	86.4	82
	2012	2012	42	DHA–PPQ	100.0	#
Kampot	2012	2013	42	DHA–PPQ	100.0	82
	2006	2008	42	AS–MQ	81.2	94
Kratie	2013	2014	42	DHA–PPQ	94.1	#
	2001	2001	28	AS–MQ	100.0	9
	2003	2003	28	AS–MQ	100.0	9
	2006	2006	42	AS–MQ	97.5	#
	2011	2011	42	DHA–PPQ	96.4	#
Mondulkiri	2011	2012	42	DHA–PPQ	83.3	82
	2013	2013	42	DHA–PPQ	100.0	#
	2014	2015	42	DHA–PPQ	90.0	#
Odder Meanchey	2003	2003	28	AS–MQ	97.8	9
	2012	2013	42	DHA–PPQ	62.5	12
Pailin	2012	2014	42	DHA–PPQ	51.1	85
	2002	2002	28	AS–MQ	85.7	9
	2004	2004	28	AS–MQ	90.1	9
	2004	2004	42	AS–MQ	79.3	9
	2007	2008	42	AS–MQ	95.0	11
	2007	2008	42	AS–MQ	100.0	77
	2007	2008	42	ATS–PYRO	89.8	77
Preah Vihear	2008	2009	42	DHA–PPQ	89.4	95
	2009	2010	42	DHA–PPQ	91.9	95
	2010	2011	42	DHA–PPQ	75.0	95
	2011	2011	42	AS–MQ	100.0	#
	2014	2015	42	ATS–PYRO	82.0	76
	2002	2002	28	AS–MQ	96.6	9
	2004	2004	28	AS–MQ	100.0	9
	2006	2006	42	AS–MQ	98.6	#
	2007	2007	42	AS–MQ	100.0	#
	2009	2009	42	DHA–PPQ	100.0	95
	2009	2009	42	AS–MQ	100.0	#
	2011	2011	42	DHA–PPQ	96.6	#
	2011	2012	42	DHA–PPQ	93.4	82
Pursat	2012	2013	63	DHA–PPQ	84.6	20
	2013	2014	42	DHA–PPQ	93.7	#
	2002	2002	28	AS–MQ	94.0	9
	2004	2004	28	AS–MQ	92.6	9
	2005	2005	42	AS–MQ	87.5	#
	2005	2005	28	A–L	82.4	82
	2005	2005	28	DHA–PPQ	98.2	82
	2007	2007	42	AS–MQ	87.9	#
	2008	2008	42	DHA–PPQ	98.7	95
	2010	2010	42	DHAPPQ	89.3	95
	2011	2011	42	DHAPPQ	83.3	#
	2011	2012	42	DHAPPQ	79.5	82
Rattanakiri	2012	2013	63	DHAPPQ	63.2	20
	2012	2013	42	DHAPPQ	97.5	82
	2014	2015	42	ATS–PYRO	89.8	76
	2002	2002	28	AS–MQ	100.0	9
	2004	2004	28	AS–MQ	100.0	9
	2006	2006	42	AS–MQ	96.2	#
	2009	2010	42	DHA–PPQ	100.0	95
	2010	2010	28	A–L	95.0	#
	2010	2010	42	DHA–PPQ	100.0	95
	2010	2010	42	DHA–PPQ	100.0	#
2012	2013	63	DHA–PPQ	98.4	20	

(continued)

TABLE 1

Continued

Study sites (province)	Start year	End year	Follow-up (days)	Antimalarial drug regimen	ACPR (%)	Reference
Siem Reap	2013	2013	42	DHA–PPQ	98.3	#
	2014	2014	42	DHA–PPQ	67.6	83
	2014	2015	42	DHA–PPQ	37.5	#
	2014	2015	42	DHA–PPQ	65.6	#

ACPR = adequate clinical and parasitological response (proportion, %); A–L = artemether–lumefantrine; AS–MQ = artesunate–mefloquine; ATS–PYRO = artesunate–pyronaridine; DHA–PPQ = dihydroartemisinin–piperaquine. Follow-up, the last day of follow-up according to the WHO protocol used (28, 42, or 63 days).
#National Center for Parasitology Entomology and Malaria Control (CNM) unpublished reports.

These anomalous curves presented a paradoxical increase in [³H]-hypoxanthine incorporation at PPQ concentrations above 100–200 nM, the physiological concentration of PPQ observed in the blood of patients treated with a standard 3-day course of DHA–PPQ⁸² To overcome the limitations of current assays and provide a more robust assessment of PPQ resistance, a new in vitro assay (named “Piperaquine Survival Assay” PSA) based on the same principle as the RSA was developed.⁸² This assay involves exposing tightly synchronized culture-adapted parasites (in vitro format) or parasites directly collected from patients (ex vivo format) to a pharmacologically relevant dose of PPQ (200 nM for 48 hours), to mimic in vivo conditions. The in vitro PSA was found to discriminate efficiently between PPQ-resistant and sensitive parasites, with resistance defined as a survival rate in the PSA ≥ 10% (defined as the cutoff for resistance on the basis of results for the isolates of patients with recrudescence infection). Patients harboring parasites with K13 mutants with an ex vivo survival rate in the PSA ≥ 10% had a 32 times higher risk of having recrudescence infection. The use of the PSA (in absence of reliable molecular marker) together with K13 genotyping was found to be a suitable method for monitoring resistance to both artemisinin and PPQ, in areas in which DHA–PPQ is currently the recommended first-line treatment and may be a reliable platform for identifying molecular markers associated with PPQ resistance and for evaluating alternative drugs effective against PPQ-resistant parasites.

Toward a molecular marker for PPQ resistance. Studies conducted in Cambodia and Thailand with the aim of characterizing molecular signatures associated with PPQ resistance have found no association between the in vitro PPQ susceptibility of *P. falciparum* isolates and copy number variations (CNVs) for the PF3D7_0520500 and PF3D7_0520900 genes located on chromosome 5 previously identified by Eastman and others.^{85–87} Spring and others recently reported a significant correlation between DHA–PPQ treatment failure and the presence of a triple-mutant haplotype for the propeller domain of the *K13* gene and for positions 1718319 and 688956 on chromosomes 13 and 10, respectively (MAL13: 1718319 and MAL10: 688956), previously reported to be associated with delayed parasite clearance in genome-wide association studies.^{42,84,88} Patients harboring triple-mutant parasites had a 5.4 times higher risk of treatment failure and a higher IC₅₀ for PPQ (33.6 nM for the triple mutant versus 24 nM for other parasites). However, rather than being directly associated with PPQ resistance (no causal impact and no biological rationale), these markers may be associated with a particular artemisinin genetic background favoring the acquisition of resistance to PPQ.⁸⁹

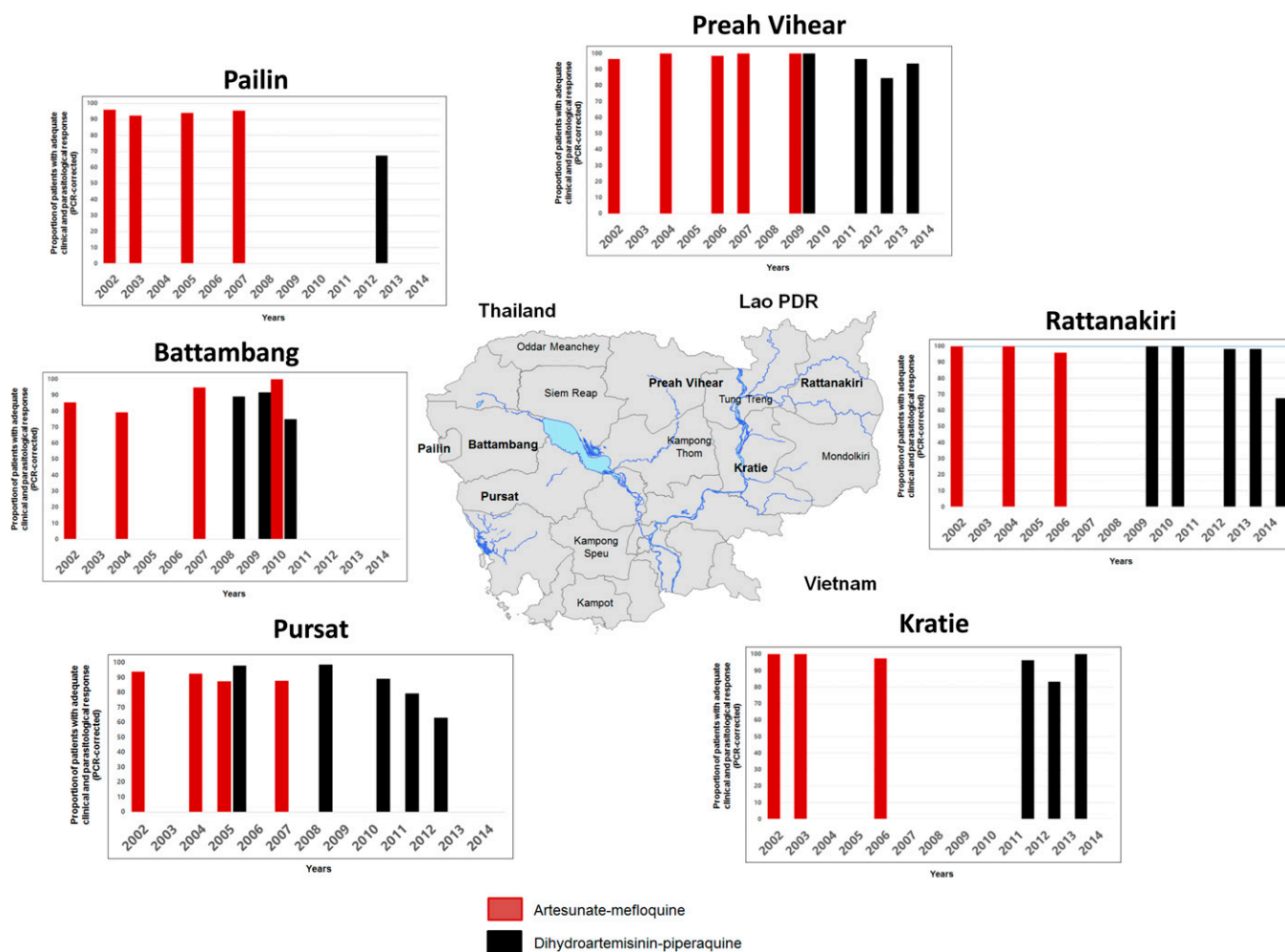


FIGURE 2. Spatial distribution and evolution of the efficacy of artesunate-mefloquine and dihydroartemisinin-piperazine 3-day standard course in different sites in Cambodia between 2001 and 2014. Details are provided in Table 1.

However, several groups with different *in vitro* assays showed that parasites with single-copy *Pfmdr-1* (and consequently a low IC_{50} value for MQ) were strongly linked to PPQ resistance.^{20,82,84,86} For instance, it has been observed that all *in vitro* PPQ-resistant isolates had a single *Pfmdr-1* copy but the converse was not true, as not all isolates with a single copy of *Pfmdr-1* were *in vitro* PPQ resistant. This finding strongly supports recent recommendations for the use of artesunate plus MQ as an alternative first-line treatment in provinces in which DHA-PPQ is failing.^{20,82,84,86} However, further investigations are required to determine whether the elimination of parasites with multiple copies of *Pfmdr-1* has resulted from the removal of MQ pressure or the selective pressure exerted by PPQ. This last finding also suggests that PfMDR1, a pump involved in the intraparasitic influx/efflux of metabolites or drugs, may play a significant role in parasite susceptibility to PPQ. For instance, PfMDR1 may play a significant role in the influx of PPQ into the digestive vacuole, in which PPQ acts on its target to kill parasites. Nonetheless, some parasites sensitive to PPQ harbor a single copy of *Pfmdr-1*, highlighting the need to find a specific molecular marker.

Several studies have also observed that specific mutations of the *Pfcr* gene were significantly associated with PPQ resistance.^{82,90} For instance, isolates containing parasites with a

variant of the Dd2 *Pfcr* allele carrying either 97Y, 343L, or 353V have higher median PSA survival rates than those harboring the Dd2 allele.⁸² In French Guiana, Pelleau and others also found that the acquisition of the C350R substitution in the *Pfcr* gene decreased susceptibility to PPQ, suggesting that PPQ pressure drove the expansion of this allele.⁹⁰ As the 353V mutation (frequently observed in Cambodia) is located close to 350R, further investigations, such as genome editing, should be carried out to assess the role of *Pfcr* mutations in the genetic background of Cambodian parasites.

Perspectives. The development of the PSA as an efficient screening tool for PPQ-resistant parasites is a major advance that may facilitate the identification of a specific molecular marker. Molecular signatures (CNV and SNP) associated with *in vitro* resistance to PPQ are being investigated by the whole-exome sequencing of PPQ-resistant (PSA survival rates $\geq 10\%$) and PPQ-susceptible (PSA survival rates $< 10\%$) parasites. In parallel, we repeatedly exposed parasites to 200 nM PPQ, over increasingly long exposure times (from 48 to 96 h), over a period of 1 year (Duru V, Witkowski B, Khim N, and Menard D, unpublished work). This resulted in the generation of a Cambodian PPQ-resistant culture-adapted parasite (PSA survival rate of 21%). The survival rates in the PSA of the initial parasite and its sibling clone cultured under the

same conditions but without the drug were estimated at 3% and 0.7%, respectively. The differences between the three exomes is analyzed, and the results obtained should facilitate accurate definition of the molecular signatures associated with PPQ resistance, while this approach has already been successfully used to identify mutations in the propeller domain of the *K13* gene as a major determinant of artemisinin resistance.

CONCLUDING REMARKS

Assessments of antimalarial drug efficacy include clinical drug efficacy studies, *in vitro* assessments of drug sensitivity, and molecular markers (Figure 3). Drug efficacy studies (clinical phenotype) remain the gold standard to evaluate the efficacy of antimalarial drugs. Clinical studies investigate both the parasite clearance time in the first 3 days of treatment to detect artemisinin resistance and the treatment failure rate (evaluated during the follow-up usually until day 42 or day 63 according to the half-life of the partner drugs) to detect partner drug resistance. *In vitro* phenotype assessing the sensitivity of *P. falciparum* malaria parasites to antimalarial drugs is also frequently used to complement data from clinical studies. These assays contribute to detect early signs of emergence of drug resistance. *In vitro* assays are also useful for drug screening and for validating candidate molecular markers associated with drug resistance. Indeed, data obtained from *in vitro* assay are directly associated to the parasite drug susceptibility and contrarily to *in vivo* phenotypes, are not affected by host factors, such as acquired immunity, bioavailability, or pharmacokinetics of antimalarial drugs. Classical *in vitro* assays (assessing IC_{50} values) have limited sensitivity for detecting resistant parasites to artemisinin and PPQ and the design of such assays needs to be adapted according to the pharmacokinetic/pharmacology profiles of the tested drugs. The RSA and the PSA are excellent examples. Finally, validated molecular markers associated to antimalarial drug resistance are relevant

to detect and monitor in real time the geospatial distribution of resistant parasites. They are useful to anticipate risks associated with the emergence and the spread of antimalarial parasite resistance and to inform policy makers. The discovery of the molecular signatures associated to antimalarial drug resistance is challenging. However, at present, next-generation sequencing of well-characterized parasites, which allow to detect SNPs and CNV differences between sensitive and resistant parasites, greatly facilitate the genomic investigations (Figure 3).

Resistance to both artemisinin and PPQ is now prevalent in Cambodia, as demonstrated by the worrying increase in the number of cases of DHA–PPQ treatment failure. The decrease in ACT efficacy has resulted from adaptation of the parasite to both artemisinin derivatives and the partner drug. Our understanding of artemisinin resistance has progressed considerably, due, in particular, to the discovery of *K13* polymorphisms. Evaluations of the global distribution of artemisinin resistance, with the screening of thousands of blood samples for the detection of *K13* mutations, are underway. However, greater efforts are required concerning PPQ resistance. There is an urgent need to characterize PPQ resistance in more detail and to identify a reliable and specific molecular marker of this resistance, as this drug is a potential partner drug candidate for inclusion in combination with the next generation of antimalarial drugs, such as ozonides, spiroindolones, and imidazolopiperazines.⁹¹

The management of falciparum malaria cases in Cambodia remains challenging. New strategies (even short-term approaches) using existing antimalarial drugs are required. These approaches may include a rotation of different ACTs (DHA–PPQ and AS–MQ), which could be effective due to the contrasting susceptibility profiles of PPQ- and MQ-resistant parasites. However, such strategies would be expected to fail in the long term, with the emergence of parasites resistant to both PPQ and MQ. This strategy is currently implemented in several provinces in Cambodia in which high failure rates have been

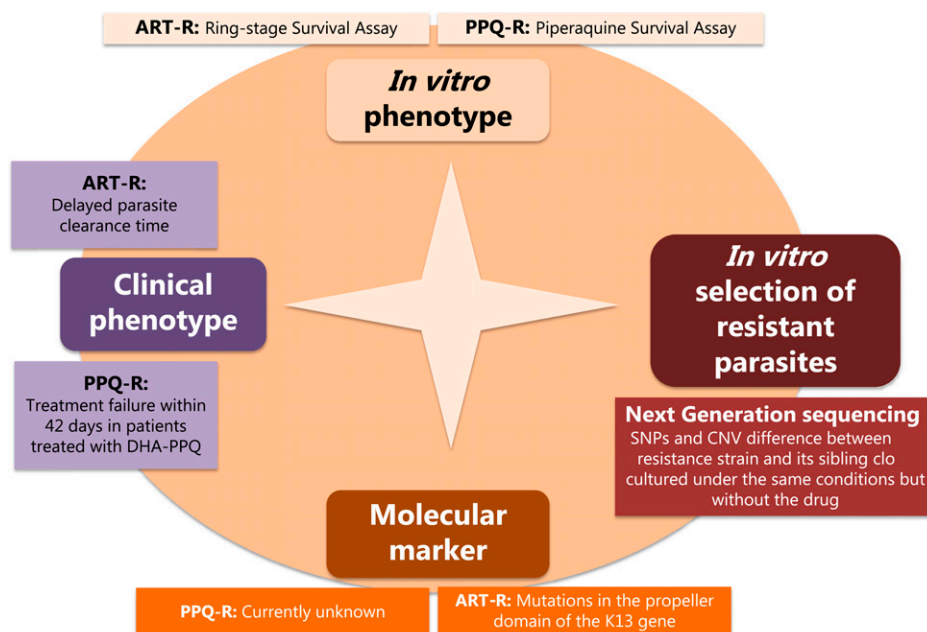


FIGURE 3. Main tools and strategy required for the detection of the molecular signatures associated with antimalarial drug resistance.

recorded for DHA-PPQ treatment. Another option is extending the course of treatment to 5 or 7 days, but it may be difficult to ensure compliance with a longer course of treatment. Finally, another alternative approach would involve combining artemisinin derivatives with two slowly eliminated partner drugs in a 3-day triple ACT, in a strategy similar to those already used for HIV and tuberculosis. This approach could involve the use of artemether-lumefantrine-amodiaquine and DHA-PPQ-MQ combinations, which are currently under evaluation for the treatment of uncomplicated falciparum malaria in areas of resistance to both artemisinin and the partner drug in conventional ACT.

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