The spread of artemisinin-resistant *Plasmodium falciparum* in the Greater Mekong subregion: a molecular epidemiology observational study

Mallika Imwong, Kanokon Suwannasin, Chanon Kunasol, Kreepol Sutawong, Mayfong Mayxay, Huy Rekol, Frank M Smithuis, Tin Maung Hlaing, Kyaw M Tun, Rob W van der Pluijm, Rupam Tripura, Olivo Miotto, Didier Menard, Mehul Dhorda, Nicholas P J Day, Nicholas J White, Arjen M Dondorp

Summary

Background Evidence suggests that the *PfKelch13* mutations that confer artemisinin resistance in falciparum malaria have multiple independent origins across the Greater Mekong subregion, which has motivated a regional malaria elimination agenda. We aimed to use molecular genotyping to assess antimalarial drug resistance selection and spread in the Greater Mekong subregion.

Methods In this observational study, we tested *Plasmodium falciparum* isolates from Myanmar, northeastern Thailand, southern Laos, and western Cambodia for *PfKelch13* mutations and for *Pfplasmepsin2* gene amplification (indicating piperaquine resistance). We collected blood spots from patients with microscopy or rapid test confirmed uncomplicated falciparum malaria. We used microsatellite genotyping to assess genetic relatedness.

Findings As part of studies on the epidemiology of artemisinin-resistant malaria between Jan 1, 2008, and Dec 31, 2015, we collected 434 isolates. In 2014–15, a single long *PfKelch13* C580Y haplotype (–50 to +31·5 kb) lineage, which emerged in western Cambodia in 2008, was detected in 65 of 88 isolates from northeastern Thailand, 86 of 111 isolates from southern Laos, and 14 of 14 isolates from western Cambodia, signifying a hard transnational selective sweep. *Pfplasmepsin2* amplification occurred only within this lineage, and by 2015 these closely related parasites were found in ten of the 14 isolates from Cambodia and 15 of 15 isolates from northeastern Thailand. C580Y mutated parasites from Myanmar had a different genetic origin.

Interpretation Our results suggest that the dominant artemisinin-resistant *P falciparum* C580Y lineage probably arose in western Cambodia and then spread to Thailand and Laos, outcompeting other parasites and acquiring piperaquine resistance. The emergence and spread of fit artemisinin-resistant *P falciparum* parasite lineages, which then acquire partner drug resistance across the Greater Mekong subregion, threatens regional malaria control and elimination goals. Elimination of falciparum malaria from this region should be accelerated while available antimalarial drugs still remain effective.

Department of Molecular Tropical Medicine and Genetics, (M Imwong PhD), **and Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine** (M Imwong, K Suwannasin BSc, C Kunasol MSc, R W van der Pluijm MD, R Tripura MD, O Miotto PhD, Prof N P J Day FRCP, Prof N J White FRS, Prof A M Dondorp MD), **Mahidol University, Bangkok, Thailand; Buntharik Hospital, Amphoe Buntharik, Ubon Ratchathani, Thailand** (K Sutawong MSc); **Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (LOMWRU), Vientiane, Laos** (M Mayxay MD); **Faculty of Postgraduate Studies, University of Health Sciences, Vientiane, Laos** (M Mayxay); **Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom** (F M Smithuis MD, R W van der Pluijm, Prof N P J Day, Prof N J White, Prof A M Dondorp); **National Centre for Parasitology, Entomology, and Malaria Control (CNM), Phnom Penh, Cambodia** (H Rekol MD); **Myanmar Oxford Clinical Research Unit, Yangon, Myanmar** (F M Smithuis, K M Tun MD); **Medical Action Myanmar, Yangon, Myanmar** (F M Smithuis); **Defence Services Medical Research Centre,**

Introduction

Artemisinin combination therapies (ACTs) have become the cornerstone of the treatment of falciparum malaria throughout the malaria endemic world.[1] New antimalarial compounds will not become widely available for several more years.[2] The emergence and geographic spread of artemisinin resistant *P falciparum* in the Greater Mekong subregion (GMS) represents a serious threat to global malaria control and to aspirations to eliminate malaria. Artemisinin resistance results in reduced ring stage susceptibility and manifests as slow parasite clearance.[3,4] As a result, fewer malaria parasites are killed during the 3 days of ACT treatment, leaving more for the partner drug to remove. This action increases the risk of treatment failure and facilitates selection of partner drug resistance.[2] Resistance to both components of the ACT

results in substantial reductions in cure rates. ACTs containing mefloquine and piperaquine are now failing across increasing areas of the GMS.[5–8]

Retrospective genotyping of archived samples showed presence of the artemisinin resistance marker at the beginning of the millennium. The *PfKelch13* mutations that mediate artemisinin resistance appear to have arisen independently on multiple occasions.[9,10] Some have taken this to suggest that a radical firewall approach to prevent the spread of these mutations might not work, and so prompted abandonment of containment and the launch of a regional malaria elimination effort in the GMS.[11,12] On the other hand, the spread of artemisinin resistance has been geographically contiguous, and there is no evidence yet for independent emergence of resistance in India or Africa.[4,13] Resistance in *P falciparum* to the antimalarials

Naypyitaw, Myanmar
(T M Hlaing MD, K M Tun)**;
Wellcome Trust Sanger Institute,
Hinxton, UK** (O Miotto)**; Malaria
Molecular Epidemiology Unit,
Institut Pasteur in Cambodia,
Phnom Penh, Cambodia**
(D Menard PhD)**; and Worldwide
Antimalarial Resistance
Network (WWARN), Bangkok,
Thailand** (M Dhorda PhD)

Correspondence to:
Dr Mallika Imwong, Department
of Molecular Tropical Medicine
and Genetics, Faculty of Tropical
Medicine, Mahidol University,
Bangkok, 10400, Thailand
noi@tropmedres.ac

Research in context

Evidence before this study

We searched PubMed on Nov 9, 2016, using the terms "artemisinin resistance", and "genotype" and "Cambodia", "Thailand", "Myanmar" and "Laos" without any date or language restrictions. This search identified 47 articles, of which 23 contained information on the epidemiology of artemisinin-resistant genotypes. Phyo and colleagues described the evolution of artemisinin resistance on the Thailand-Myanmar border, and how the E252Q *PfKelch* mutation predominated initially but was overtaken in recent years by C580Y as artemisinin combination therapies (ACTs) began to fail. Takala-Harrison and colleagues conducted a genome-wide association study which showed multiple independent emergences of *PfKelch13* mutations in *Plasmodium falciparum* populations in southeast Asia, but haplotype analysis also suggested spread of the Y493H and C580Y mutations from Cambodia across the border to Vietnam. Menard and colleagues assessed the genetic relatedness of *PfKelch13* mutant parasites in the Greater Mekong Subregion (GMS) and also identified "numerous emergence events alongside spreading of a small group of mutations for artemisinin resistance".

Added value of this study

This longitudinal study conducted over 8 years in four countries has two important findings; that over time single lineages of artemisinin-resistant parasites dominate, outcompeting the other parasites, and then under continued ACT therapies drug pressure, spreads—in one case across three countries—and that artemisinin resistance selects for piperaquine resistance.

Implications of all the available evidence

This study shows that artemisinin resistance has continued to evolve such that with time, and continued selection pressure, what started as multiple independent emergences of *PfKelch13* mutations was overtaken by a few dominant lineages that spread and acquired partner drug resistance. This same sequence of events occurred previously when antimalarial drug resistance emerged in the GMS and later spread to Africa. Malaria elimination efforts need to be accelerated in the GMS while antimalarial drugs remain effective, particularly in areas where these successful dominant *P falciparum* lineages are found.

chloroquine and sulfadoxine-pyrimethamine also arose on multiple occasions in the GMS and in the case of pyrimethamine, resistance eventually a single parasite lineage dominated (a so-called hard selective sweep) and it was this dominant resistant lineage that spread across India and Africa.[14,15] Using microsatellite genotyping around the *PfKelch13* gene in *P falciparum* isolates collected over 7 years from four countries in the GMS, we test the hypothesis that history is being repeated—ie, that dominant lineages of artemisinin-resistant falciparum malaria parasites are now spreading through the GMS. Using molecular genotyping we also investigate the relation between artemisinin resistance (*PfKelch13* mutation)[4,9,10,13] and piperaquine resistance (*Pfplasmepsin2* amplification)[16,17] and we discuss the implications of the findings for the regional and global containment of multidrug-resistant falciparum malaria.

Methods

Sample collection

As part of studies on the epidemiology of artemisinin-resistant malaria (reported previously[3,4,18,19] and ongoing; trial registration numbers NCT01350856, NCT02453308), we collected 434 blood spots from patients with microscopy or rapid test confirmed uncomplicated falciparum malaria from Pailin, Cambodia (113, 2008, 2011, and 2015); Champasak, Laos (111, 2013–14), Ubon Ratchathani (88, 2014–15) and Phusing (15, 2015) in Thailand; and Kyainseikgyi, Kayin, Myanmar (107, Jan 1, 2014, to Dec 31, 2015). Approvals for these studies were obtained from the ethical review boards of

the Faculty of Tropical Medicine, Mahidol University (TMEC 12-046); Department of Medical Research, Ministry of Health and Sports (Myanmar); The Defence Services Medical Ethics Committee (Myanmar); the Ministry of Health in Cambodia; and the Oxford Tropical Medicine Ethics Committee. We obtained blood spot blood samples on filter paper (Whatman 3MM, GE Healthcare, UK) and kept samples dry at room temperature until DNA extraction. We did DNA purification using a QIAgen Kit (QIAgen, Hilden, Germany) according to the manufacturer's instructions.

Assessment of mutations in *PfKelch13*

We sequenced nested PCR products of the full length of the *PfKelch13* gene (2181 bp including one exon),[4] with an ABI Sequencer (Macrogen Inc, Seoul, Korea). Cross contamination was monitored by adding negative control samples in every run. We aligned sequencing results against *PfKelch13* of reference strain 3D7 (putative 9PF13_0238 NCBI Reference Sequence [3D7]: XM_001350122.1), using Bioedit software (Abbott, Carlsbad, CA, USA). Polymorphic patterns were assessed by two people blinded to the origin of the sample.

Pfplasmepsin2 gene amplification

We quantified *Pfplasmepsin2* copy number using relative-quantitative RT-PCR based on SYBR-Green fluorescence on an Applied Biosystems ViiA 7Real-Time-PCR-System[16] (Life technologies, Carlsbad, CA, USA). We did amplification in triplicate on a total volume of 20 µL as singleplex PCR using SsoFast

Evagreen Supermix (BIO-RAD, Hercules, CA, USA). Every amplification run contained three replicates of 3D7 calibrators and triplicates without template as negative controls. β-*tubulin* served as an internal standard for the amount of sample DNA added to the reactions. We calculated copy numbers using the formula: copy number=$2^{-\Delta\Delta Ct}$ with $\Delta\Delta C_t$ denoting the difference between ΔC_t of the unknown sample and ΔC_t of the reference sample.

Microsatellite genotyping of the regions flanking *PfKelch13*

Microsatellite genotyping of the −56 kb to +72 kb regions flanking the *PfKelch13* gene was done as described previously.[20,21] For isolates carrying the *PfKelch13* C580Y allele, we designed seven additional microsatellite markers covering −247 kb to +269 kb and for the *PfKelch13* R539T allele 17 additional microsatellites covering −579 kb to +631 kb (appendix). We did single PCR for 35 cycles of 1 minute at 95°C, 1 minute annealing at 55°C, and 1 minute extension at 72°C. The lengths of the PCR-generated products were measured by comparison with internal size standards (Genescan 500 LIZ) on an ABI 3100 Genetic analyser, using Genescan and Genotyper software (PE Applied Biosystems) to measure allele lengths and to quantify peak heights. Negative control samples were included to monitor cross-contamination. We analysed a subset of ten samples in triplicate to assess intra-assay consistency. 79 samples with multiple genotypes were included in all analyses except for the assessments of genetic relatedness.

Additional assessments of genetic diversity

To assess genetic relatedness in parasites with the long *PfKelch13* haplotypes and between *Pfplasmepsin2* amplified and non-amplified parasites, we genotyped parasite isolates for variable blocks within the *msp1, msp2,* and *glurp* genes,[22] and for nine microsatellites across the genome[23] using semi-nested PCR. Genetic similarity between isolates was analysed by unweighted pair-group method with arithmetic average (Unweighted Pair Group Method with Arithmetic Mean [UPGMA]) using BioNumerics software version 7.5 (Applied Maths, Sint-Martens-Latem, Belgium).

Analysis

We examined genetic variation at each microsatellite locus or heterozygosity (H_e) using the formula $H_e=[n/(n-1)][1-\sum p_i^2]$, and calculated the sampling variance of H_e as $2(n-1/n^3[2(n-2)]\sum p_i^3 - \sum p_i^2)^2$ where n is the sample size and p_i is the frequency of the i-th allele. We constructed a median-joining network diagram tree assessing genetic relatedness between parasite isolates with different *PfKelch13* alleles, based on nine microsatellite markers spaced at −56 and +72 kb intervals in parasite isolates from Pailin from 2008, 2012, and 2015, using Network software version 5. Heterozygosity at each location was compared with wild type by Mann-Whitney *U* test.

See **Online** for appendix

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

From Jan 1 to Dec 31, 2008, in Pailin, western Cambodia, where artemisinin resistance was first described, we identified *PfKelch13* gene propeller region point mutations in 50 of 59 isolates: C580Y in 26 of 59 isolates, Y493H in 13 of 59 isolates, R539T in eight of 59 isolates, other *PfKelch13* mutations in three of 59 isolates, and wild-type *PfKelch13* was found in nine of 59 isolates. The C580Y allele was detected in 37 of 40 isolates in 2011, and 14 of 14 isolates in 2015, whereas R539T was detected in one of 40 isolates in 2011, and none of 14 in 2015.

In 2015, in Phusing, northeastern Thailand, approximately 200 km northeast of Pailin, all 15 isolates

	Total	*PfKelch* with single *Pfplasmepsin 2* copy												*PfKelch* C580Y with multiple *Pfplasmepsin 2* copies
		Wild type	C580Y	R539T	F446I	G449A	N537I	R561H	E252Q	A481V	D584V	Y493H	Other mutations*	
Pailin, Cambodia 2008–09	59	9	14	8	0	0	0	0	0	2	1	13	0	12
Pailin, Cambodia 2011–12	40	2	22	1	0	0	0	0	0	0	0	0	0	15
Ubon Ratchathani, Thailand 2014	88	6	64	17	0	0	0	0	0	0	0	0	0	1
Champasak, Laos 2014	111	12	86	13	0	0	0	0	0	0	0	0	0	0
Pailin, Cambodia 2015	14	0	4	0	0	0	0	0	0	0	0	0	0	10
Phusing, Thailand 2015	15	0	0	0	0	0	0	0	0	0	0	0	0	15
Kayin, Myanmar 2013–15	107	55	6	0	15	7	4	4	1	0	0	0	15	0

*Other *PfKelch* mutations were C469F, K438N, P441L, P527H, P553L, P574L, P667R, and R575K.

Table: Frequency distribution of *PfKelch13* gene mutations and gene amplification of *Pfplasmepsin2* in four countries of the Greater Mekong subregion 2008–15
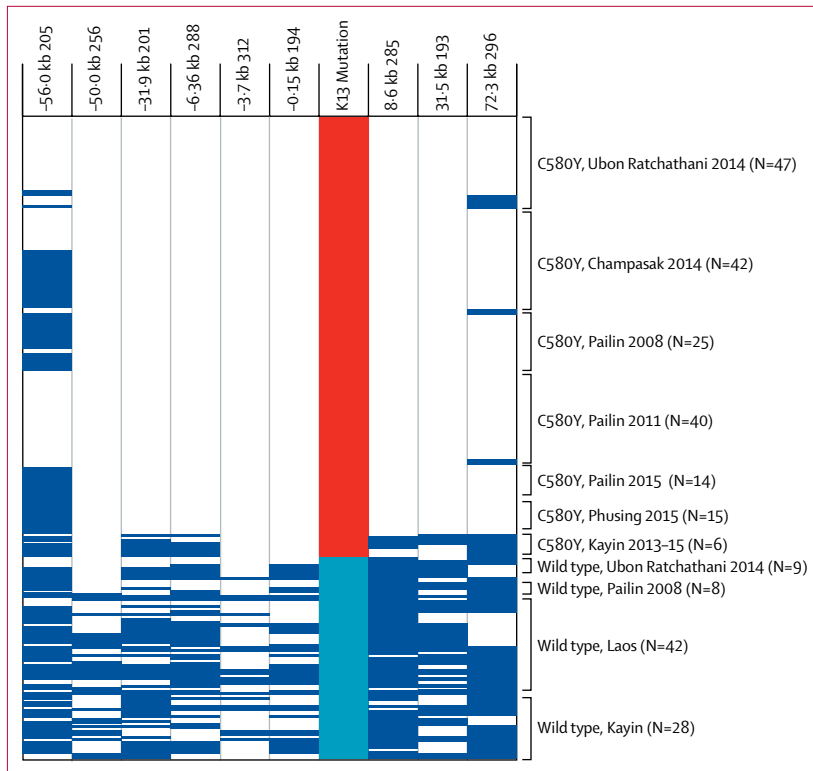
Figure 1: Microsatellite haplotypes of the *PfKelch13* flanking regions
Red box=C580Y. Light blue box=wild type. Each row represents one parasite isolate; white cells indicate identical microsatellite alleles compared with the most frequent allele and dark blue cells indicate differences.

[SE] 0·059) in wild type. Excluding the isolates from Myanmar, over 800 km to the west, mean H_e was 0·687 (SE 0·058) in wild-type, compared with 0·17 (0·11) in C580Y isolates from Pailin 2011–15, p=0·0002; 0 (0) in Phusing, Thailand, p=0·0002; 0·046 (0·031) in Ubon Ratchathani, Thailand, p=0·0002; and 0·059 (0·053) in Champasak, Laos, p=0·0003. The valley of reduced diversity was asymmetric in size between the 5′ and 3′ sides of the gene and was similar between isolates from Cambodia, Laos, and Thailand (appendix). *Pfplasmepsin2* amplification was observed only in parasites with this dominant *PfKelch13* C580Y long haplotype (53 of 53 isolates). Comparison of the nine microsatellite defined *PfKelch13* C580Y haplotypes of isolates from the four countries showed that the C580Y allele found in southeastern Thailand (Phusing and Ubon Ratchathani) and southern Laos (Champasak) shared a common origin with the C580Y alleles observed in Pailin, but was genetically remote from the Myanmar haplotype (figure 1). Between 2008 and 2014, in Pailin (western Cambodia), the overall mean H_e of the microsatellite defined *PfKelch13* haplotypes reduced from 0·496 (SE 0·005) to 0·158 (0·020; p=0·0002), suggesting a selective sweep through the parasite population. Parasites with the same C580Y haplotype were not clonal since there was significant diversity in microsatellites elsewhere in the genome and in the polymorphic loci in the *msp1, msp2,* and *glurp* genes that showed an overall mean H_e of 0·529 (SE 0·030) for three loci combined (individual values: 0·680 [0·040] for *glurp*, 0·453 [0·316] for *msp1*, and 0·457 [0·019] for *msp2*).

Microsatellite defined *PfKelch13* R539T haplotypes from 39 parasite isolates from Cambodia, Laos, and Thailand were compared with the 87 *PfKelch13* wild-type isolates. Mean H_e in wild-type isolates was 0·691 (SE 0·190) compared with 0·077 (0·005) in R539T parasites (p<0·0001), signifying much reduced microsatellite diversity −50 to +242 kb around the R539T allele. The conserved region around the R539T allele was asymmetric, and was larger than in the C580Y haplotype. The R539T haplotype was the same in isolates from Ubon Ratchathani and Champasak, and was only a single locus different from those in Pailin, suggesting a common origin (appendix). Nine microsatellite markers flanking the *PfKelch13* F446I allele were genotyped in six samples from Homalin, Sagaing and 20 samples from Kyainseikgyi, Kayin, Myanmar. A unique identical *PfKelch13* haplotype was observed in Kayin with a mean H_e 0 (SD 0), which was not observed elsewhere. By comparison, the mean H_e assessed from 45 local *PfKelch13* wild-type isolates was 0·599 (SE 0·010), signifying high diversity.

A median-joining network tree was generated using microsatellite defined haplotypes around *PfKelch13* from 346 *P falciparum* isolates obtained from the four countries (appendix). There was strong genetic clustering of the C580Y allele in parasites from Cambodia, Thailand, and Laos, far from the dispersed haplotypes observed in

collected contained the C580Y mutation. Over the same time period in 2015, the C580Y allele was present in 64 of 88 isolates from Ubon Ratchathani district (about 100 km northeast of Phusing), and 17 of 88 isolates contained the R539T allele. In 2014, in Champasak, southern Laos, approximately 350 km northeast of Pailin, 86 of 111 parasite isolates contained the C580Y allele and 13 of 111 isolates contained the R539Y allele. By contrast, in 2015, in Kyainseikgyi, Kayin, Myanmar, about 600 km northwest of Pailin, the C580Y mutation was present in only six of 107 samples (table).

In Pailin, *Pfplasmepsin2* amplification was observed in 12 of 59 isolates in 2008, 15 of 40 isolates in 2011, and ten of 14 isolates in 2015. In Phusing, all 15 isolates from 2015 displayed amplification. The median copy number was 0·98 (range 0·75–3·27) in 2008 and 1·65 (0·66–3·41) in 2015 in Pailin, and 1·93 (0·55–3·99) in 2015 in Phusing. By contrast, amplified *Pfplasmepsin2* was found *i*n only one of 88 isolates from Ubon Ratchathani, none of 111 isolates from Champasak, and none of 107 from Myanmar.

Comparison of microsatellite haplotypes in 269 isolates carrying C580Y with 84 with *PfKelch13* wild types from the same countries showed a sharp reduction in micro-satellite variation spanning a −50 to +72 kb region around the *PfKelch13* gene. Mean H_e was 0·688 (standard error

northern and southern Myanmar and from local parasites containing wild type or the F446I allele. Clustering was also observed among isolates containing the R539T allele in samples from Cambodia, Thailand, and Laos.

Using microsatellite typing across the genome, relatedness between the 49 isolates with concurrent *Pfplasmepsin2* amplification was compared with the 40 *Pfplasmepsin2* single copy isolates but the same *PfKelch13* C580Y long haplotype. Overall mean H$_e$ of the *Pfplasmepsin2* amplified isolates was significantly lower than that of single copy isolates (p=0·021) and cluster analysis by UPGMA showed that isolates containing *Pfplasmepsin2* amplification diverged genetically from single copy isolates (appendix). Isolates with *Pfplasmepsin2* amplification obtained from Pailin in 2008 and 2011 clustered separately from *Pfplasmepsin2* amplified isolates obtained from Pailin and Phusing in 2015.

Discussion

Artemisinin resistance in *P falciparum* is evolving in the GMS. This study suggests that the previously described soft selective sweeps,[9,10] which resulted from the multiple independent emergences of mutant *PfKelch13* alleles, have now been superseded by hard selective sweeps by presumably fitter parasites. A single long haplotype *PfKelch13* C580Y mutant parasite lineage has now spread across three countries. The dominant haplotype prevalent in 2014 and 2015 in both northeastern Thailand (Ubon and Srisaket provinces) and southern Laos (Champasak province) is the same as that observed from 2008 onwards in western Cambodia, indicating a common origin. Given the time course of artemisinin resistance in the region,[4] and the low prevalence of falciparum malaria in northeastern Thailand 8 years ago, this suggests resistance spread from western Cambodia to Laos and Thailand, probably along with the cross-border movement of people (figure 2). *Pfplasmepsin2* amplification, which is one of the genes associated with piperaquine resistance,[16,17] has emerged on the back of the *PfKelch13* C580Y mutation, and this multidrug-resistant *P falciparum* lineage is spreading widely, causing high ACT failure rates. From 2008 to 2015 in Pailin, western Cambodia, where artemisinin resistance was first described, parasites containing the C580Y mutation have now replaced parasites containing other *PfKelch13* mutations, including the temporarily successful R539T haplotype. Malaria incidence has fallen, so this represents a relative rather than an absolute increase.

Earlier genetic epidemiological analysis from study sites in the GMS east of Bangkok showed clustering of long haplotypes around the *PfKelch13* C580Y allele, consistent with a common origin in different subpopulations across Cambodia and some samples from Vietnam and eastern Thailand.[10] Our study uses microsatellite genotyping and shows further selection and reduction in variability over time, with a common origin of *PfKelch13* C580Y parasite
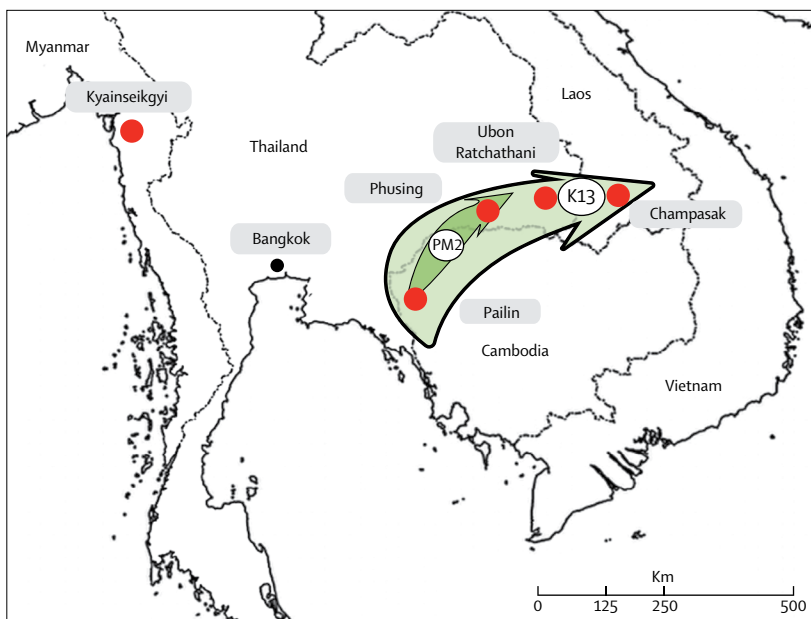

Figure 2: Study sites
A single artemisinin-resistant *Plasmodium falciparum PfKelch13* C580Y lineage (K13) has spread over an arc involving Cambodia, Thailand, and Laos. *Pfplasmepsin2* amplification (PM2), has emerged subsequently within this lineage. Red dots represent location of the study sites.

isolates sharing a similar long haplotype in all isolates surveyed from Cambodia and adjacent countries, by contrast with the high diversity in the flanking regions of parasites carrying wild-type *PfKelch13*. A similar trend, in which C580Y mutant parasites overtook other *PfKelch13* propeller region mutations, has been observed on the Thailand–Myanmar border.[7] The C580Y mutation does not confer a higher level of artemisinin resistance than many other *PfKelch13* mutations (including R539T), but this lineage appears to be fitter or more transmissible. *PfKelch13* mutations are found throughout the malaria endemic world, but artemisinin resistance has emerged and spread only in the GMS. Genome-wide association studies have shown that in addition to the *PfKelch13* mutations, a combination of backbone mutations are closely linked to the artemisinin-resistant phenotype and are present in association with different *PfKelch13* variants.[24] These other genetic changes presumably confer an additional fitness advantage, facilitating selection and spread.

The emergence and spread of piperaquine resistance in Cambodia is a sinister development. More than half of the parasite isolates obtained from Pailin in Cambodia and Phusing in nearby Thailand in 2015 also carried a marker for piperaquine resistance; gene amplification of the *Pfplasmepsin2* genes on chromosome 14.[16,17] Until 2016, dihydroartemisinin-piperaquine was the first-line ACT in Cambodia, but its efficacy has decreased markedly since 2008.[5,6,8,25] High treatment failure rates facilitate the spread of resistance. *P falciparum* isolates from northeastern Thailand also carried the piperaquine

resistance marker, yet first-line treatment there has been artesunate-mefloquine, which suggests recent importation rather than local selection. The *Pfplasmepsin2* amplified parasites all carried the same *PfKelch13* C580Y long haplotype, but were less diverse than the *Pfplasmepsin2* single copy isolates with this haplotype, which suggests that *Pfplasmepsin2* amplification occurred after the selection of the successful *PfKelch13* C580Y lineage.

A limitation of the study is that geographic spread to other areas of the GMS was not investigated. Assessment of parasite diversity was also compromised by the low prevalence now of wild-type *PfKelch13* in some study sites. However, this does not challenge the main conclusion of the study: parasites originating from artemisinin resistance soft selective sweeps[26] have been replaced by a single dominant genotype (a hard sweep) that is spreading widely. This change occurred previously with antifolate resistance in *P falciparum*.[15] *Pfdhfr* mutant genotypes (including the triple 108, 51, and 59 genotype) emerged de novo on multiple occasions but over time a single triple genotype parasite lineage dominated in the GMS and this spread across continents.[15] It is also possible that the dominant *Pfcrt* K76T genotype, conferring chloroquine resistance, was preceded by multiple soft sweeps.

The findings in this study have important implications for public health and containment strategies. Artemisinin resistance in this region was clearly a prelude to partner drug resistance. The combination of artemisinin and piperaquine resistance is now spreading quickly throughout Cambodia. Fitter multidrug-resistant parasites are spreading throughout western Cambodia, southern Laos, and northeastern Thailand. This evidence reinforces the urgency for *P falciparum* elimination in areas of artemisinin and partner drug resistance since containment will become increasingly difficult as drug efficacy declines. Further spread of these successful multidrug resistant parasites through India to sub-Saharan Africa would be a public health disaster. Border crossings between countries in the region are frequent, and mobile populations who carry parasites asymptomatically are a likely source of transnational spread.[27,28]

In conclusion, this study shows that over a large area of the GMS multiple independently originating *PfKelch13* mutant *P falciparum* parasites have been replaced by a single dominant long haplotype *PfKelch13* C580Y mutant lineage. This mutant had its likely origin in western Cambodia, and then spread to northeastern Thailand and southern Laos. These dominant artemisinin-resistant parasites are developing resistance to the ACT partner drugs, and are causing high rates of malaria treatment failure. What appeared initially to be an unusual pattern of multiple events has transformed into a more familiar scenario in which successful closely genetically related malaria parasites, which are highly drug resistant, spread and threaten malaria control and elimination throughout the region and beyond.

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