Approche génomique et bioinformatique de l'émergence et de la diffusion des résistances chez Plasmodium au Cambodge

Nimol Khim

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Collège Doctoral du Languedoc-Roussillon

THÈSE

Pour obtenir le grade de Docteur

Délivré par **UNIVERSTE MONTPELLIER I**

Préparée au sein de l'école doctorale **SIBAGHE**
Et de l'unité de recherche UFR SCIENCES PHARMACEUTIQUES ET BIOLOGIQUES

Spécialité : **Microbiologie et Parasitologie**

Présentée par **KHIM NIMOL**

APPROCHE GENOMIQUE ET BIOINFORMATIQUE DE L'EMERGENCE ET DE LA DIFFUSION DES RESISTANCES CHEZ *PLASMODIUM* AU CAMBODGE

Soutenue le 10 décembre 2014 devant le jury composé de

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M le Dr Hubert BARENNES	INSERM	Rapporteur
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UNIVERSITÉ MONTPELLIER I

UFR SCIENCES PHARMACEUTIQUES ET BIOLOGIQUES

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10 December 2014

GENOMIC AND BIOINFORMATIC APPROCHES TO STUDY THE EMERGENCE AND THE SPREAD OF ANTIMALARIAL DRUG RESISTANCE OF MALARIA PARASITES IN CAMBODIA

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Dr Lek DYSOLEY	Guest

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RÉSUMÉ EN FRANÇAIS (SUMMARY IN FRENCH)

Le paludisme, maladie parasitaire et vectorielle, sévissant principalement dans les zones intertropicales où vit près de 40% de la population mondiale, reste un problème majeur en santé publique. Les cinq espèces de *Plasmodium* connues infectées le paludisme chez l'homme sont présentes au Cambodge, qui est reconnu comme l'épicentre de l'émergence de souches de *P. falciparum* multi-résistantes (chloroquine, sulfadoxine- pyriméthamine, méfloquine, artémisinine), pouvant entraver les progrès accomplis depuis plus d'une décennie.

Le travail de thèse intitulé « **Approche génomique et bio-informatique de l'émergence et de la diffusion des résistances chez *Plasmodium* au Cambodge** » avait pour objectif de développer de nouveaux outils moléculaires et biologiques pour

1) une meilleure compréhension de l'impact des stratégies mises en place pour lutter contre le paludisme à *P. falciparum* sur les autres espèces de *Plasmodium*,

2) la mise en place d'outils biologique et moléculaire, permettant de mieux définir l'épidémiologie des parasites résistants, en particulier la résistance à la quinine et aux dérivés de l'artémisinine,

3) l'étude et la définition des sous-populations parasitaires circulant au Cambodge afin d'estimer les risques associés à la diffusion de la résistance à l'artémisinine.

Cette thèse a été réalisée dans l'Unité d'Epidémiologie Moléculaire du Paludisme à l'Institut Pasteur du Cambodge (IPC) sous la codirection du Dr. Didier Ménard (Chef de laboratoire à l'IP) et du Pr. Emmanuel Cornillot (Professeur à l'Université Montpellier I).

Le premier objectif visait à étudier l'impact de la pression médicamenteuse sur la dynamique d'évolution des populations parasitaires. Nous avons d'abord évalué le polymorphisme des gènes associés à la résistance à la pyriméthamine (gène *dhfr, dihydrofolate reductase*) chez *Plasmodium malariae* et *Plasmodium ovale* **(article 1 et manuscrit en préparation1)**, et le polymorphisme du gène *mdr-1* (*multidrug resistance 1*) associé à la résistance à la mefloquine chez *P. vivax* **(article 2)**. De plus, en collaboration avec l'Institut Pasteur de Madagascar, nous avons étudié le lien pouvant exister entre le polymorphisme du gène candidat *Plasmodium falciparum Na+/H+ exchanger* (*Pfnhe-1*) et la résistance (clinique et *in vitro*) de *P. falciparum* à la quinine **(articles 3 et 4)**.

Le deuxième objectif s'est interessé au développement d'outils biologiques et moléculaires permettant d'évaluer la résistance des souches de *P. falciparum* aux dérivés de l'artémisinine. Les 3 articles présentés **(articles 5, 6 et 7)** decrivent la méthodologie d'approche originale utilisée associant la génomique, la biologie, la clinique et l'épidémiologie, qui a permis d'aboutir à la découverte d'un marqueur moléculaire (mutations au sein du gène Kelch 13) fiable pour identifier les souches résistantes aux dérivés de l'artémisinine.

Le dernier objectif était consacré au développement de la technique PCR-LDR-FMA appliqué à la détection d'un panel de 24 SNPs permettant de caractériser par un « barcode » chaque isolat de *P. falciparum*. Cette technique couplée avec une analyse bio-informatique et statistique des données nous a permis d'étudier et de définir la structuration des populations parasitaires circulant au Cambodge afin d'estimer les zones à risque de diffusion de la résistance à l'artémisinine (**manuscrit en préparation 2**).

A travers ce travail de thèse, nous nous sommes efforcés de montrer la puissance des techniques de biologie moléculaire disponibles couplées avec des approches génomique et bio-informatique pour améliorer notre compréhension de la dynamique d'évolution des populations parasitaires. Ce travail s'est essentiellement concentré sur les phénomènes liés à l'émergence et de la diffusion des parasites résistants aux antipaludiques, le but final de ce travail étant d'améliorer les stratégies de lutte mises en place pour atteindre l'ambitieux objectif d'élimination du paludisme.

RÉSUMÉ EN ANGLAIS (SUMMARY IN ENGLISH)

Malaria, a protozoan vector-borne disease, is mainly prevalent in tropical areas, where nearly 40% of the world population is residing and remains one of the most concerns for public health worldwide. In Cambodia, the five *Plasmodium* species known to cause malaria in humans are present. The main feature of this country is that it is recognized as the epicenter of the emergence of multi-resistant *P. falciparum* parasites (to chloroquine, sulfadoxine-pyrimethamine, mefloquine, and artemisinin), a very significant menace to public health in the Mekong region that could impact the worldwide strategy to fight malaria.

The thesis presented here, entitled "**Genomics and Bioinformatics in the emergence and spread of resistant *Plasmodium* in Cambodia**" aimed to develop new molecular and biological tools for:

1) improving our understanding of the collateral impact of the strategies implemented to fight against *falciparum* malaria on the other *Plasmodium* species;

2) defining the molecular epidemiology of antimalarial resistant parasites, especially resistance to quinine and artemisinin derivatives;

3) studying and defining the structure of *P. falciparum* parasite populations circulating in Cambodia to estimate areas at risk of spread of artemisinin resistance, using genomic approaches and bioinformatics.

This thesis was carried out in the Malaria Molecular Epidemiology Unit at Pasteur Institute in Cambodia (IPC) under the co-direction of Dr. Didier Ménard (Head of the Unit, IP) and Pr. Emmanuel Cornillot (Professor, University of Montpellier I).

The first objective of this work was to study the impact of drug used to treat *falciparum* malaria on the dynamics of other *Plasmodium* species. In a first step, we evaluated the polymorphism in gene associated to pyrimethamine resistance (*dhfr* gene, *dihydrofolate reductase*) in *Plasmodium malariae* and in *Plasmodium ovale* **(article 1 and manuscript in preparation 1)** and the polymorphism in *mdr-1* gene (*multidrug resistance 1* gene) associated to mefloquine resistance in *P. vivax* **(article 2)**. Secondly, in collaboration with Pasteur Institute in Madagascar, we investigated the association between the polymorphism in *Plasmodium falciparum Na + / H + exchanger* gene (*Pfnhe-1*) and quinine resistance defined either by clinical or *in vitro* phenotypes **(articles 3 and 4)**.

The second objective was focused on the development of novel biological and molecular tools to assess the resistance of *P. falciparum* to artemisinin derivatives. The three papers presented **(articles 5, 6 and 7)** describe an original approach combining genomics, biological, clinical and epidemiological studies, which lead to the discovery of a molecular marker (mutations Kelch 13 gene) associated to artemisinin resistance.

The third and final objective was devoted to the development of the PCR-LDR-FMA technology applied to the detection of a panel of 24 SNPs to characterize a "barcode" of *P. falciparum* isolates. This

technic coupled with bioinformatics and statistical analysis allowed us to study and define the structure of the parasite populations circulating in Cambodia for estimating areas at risk of spread of artemisinin resistance **(manuscript in preparation 2).**

Through this work, we have tried to show the usefulness of available molecular biology methods coupled with genomic and bioinformatics approaches to improve our understanding of the dynamics of the malaria parasite populations. This work has been mainly focused on the emergence and spread of antimalarial resistant parasites, keeping in mind that the ultimate goal of this work was to improve strategies implemented to achieve the ambitious goal of malaria elimination.

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LISTE DES ABREVIATIONS

ADN : Acide désoxyribonucléique

ACT : Artemisinin-based combination therapy

BET : Bromure d'éthidium

CNM : Centre National de Malariologie

cyt b : gène codant pour le cytochrome b

dNTP : Désoxynucleotide triphosphate

EDTA : Ethylene diamine tetraacetic acid

HbE : Hémoglobine E

LDH : lactate déshydrogenase

HRP2. : Histidine Rich Protein 2

PCR : Polymerase chain reaction

PCR-LDR-FMA: Polymerase chain reaction ligase detection reaction fluorescence microsphere based assay

SDS : Sodium dodécyl sulfate

TDR : Test de diagnostic rapide

TPI : Traitement Préventif Intermitent

OMS : Organisation Mondiale de la Santé

RBM : Roll Back Malaria

SNP : Single nucleotide polymorphism

SAPE: Streptavidin-R-Phycoerythrin

Pf: Plasmodium falciparum

Pv: Plasmodium vivax

Pm: Plasmodium malariae

Po: Plasmodium ovale

Pk: Plasmodium knowlesi

Plasmodium spp.: Plasmodium sub species

INTRODUCTION

Malgré les efforts importants consentis par les organisations nationales et internationales pour mettre en œuvre des stratégies efficaces pour lutter contre le paludisme, cette maladie parasitaire reste l'une des plus répandues et un problème majeur de santé publique dans les régions endémiques. Actuellement, près de 40% de la population mondiale vit en zone d'endémie (Hay et al. 2004, Henry et al. 2009). En 2012, on estimait que le paludisme avait affecté 207 million d'individus et était responsable de 627 000 décès (WHO 2013), essentiellement en Afrique sub-saharienne et chez les enfants avec l'âge moins de 5 ans (Breman et al. 2004, Hay et al. 2005). Malgré ces effort, il nous apparait clairement, depuis plusieurs années, que les stratégies visant à contrôler la maladie ne suffiront pas pour atteindre l'objectif d'élimination fixé par l'OMS (Alonso et al. 2011) et que la mise en place d'outils innovants et plus performants sera nécessaire, en particulier pour mesurer plus précisément la réduction du poids du paludisme (en terme de morbidité et de mortalité), pour détecter précocement et efficacement les infections chez les sujets symptomatiques, pour explorer l'étendue du réservoir parasitaire chez les sujets asymptomatiques et évaluer l'intensité de la transmission, mais surtout pour préserver l'efficacité des antipaludiques.

Au Cambodge, zone de faible transmission en Asie du Sud Est, les cinq espèces de *Plasmodium* pouvant infecté l'homme sont présentes. Une des principales caractéristiques de ce pays est qu'il est l'épicentre de l'émergence de parasites ayant acquis la capacité de résister aux antipaludiques comme nous avons pu le constater avec la chloroquine, l'association sulfadoxine–pyriméthamine, la méfloquine et plus récemment avec les combinaisons thérapeutiques à base de dérivés de l'artémisinine (ACT) (Dondorp et al. 2009, Noedl et al. 2008, Saunders et al. 2014, Wongsrichanalai and Meshnick 2008). Dans ce contexte et pour éviter que les parasites résistants ne diffusent dans les zones de fortes transmission comme l'Afrique sub-saharienne comme nous l'avons connu avec les précédentes générations d'antipaludiques, il est primordial de mieux comprendre les mécanismes mis en place par les parasites pour résister aux antipaludiques et de mieux évaluer l'impact des antipaludiques sur la dynamique d'évolution des populations parasitaires (au sens large en incluant les autres espèces de *Plasmodium*).

Le but de notre projet de thèse intitulé « Approche génomique et bio-informatique de l'émergence et de la diffusion des résistances chez *Plasmodium* au Cambodge », se place donc dans ce contexte particulier où malgré la volonté de contenir les parasites résistants, d'éviter leur propagation et d'éliminer le paludisme à *Plasmodium falciparum* d'ici 2020 et celui à *Plasmodium vivax* d'ici 2025 (CNM 2011), nous devons appréhender la dynamique des populations parasites soumises à une pression de sélection sans précédent. Nos objectifs ont donc été de développer et de mettre en place de nouveaux outils, permettant d'approfondir notre connaissance sur les « paludismes » (paludismes à *P. falciparum* et *P. vivax*, mais également paludismes provoqué par d'autres espèces de *Plasmodium* émergentes) par :

1. une meilleure compréhension de l'impact des stratégies mises en place pour lutter contre le paludisme à *P. falciparum* sur les autres espèces de *Plasmodium*;

2. la mise en place d'outils biologique et moléculaire, permettant de mieux définir l'épidémiologie des parasites résistants, en particulier la résistance à la quinine et aux dérivés de l'artémisinine;

3. l'étude et la définition de la structuration des populations parasitaires circulant au Cambodge pour estimer les zones à risque de diffusion de la résistance à l'artémisinine, en utilisant des approches génomique et bio-informatique.

CONTEXTE ET SYNTHESE BIBLIOGRAPHIQUE

1. Situation du paludisme dans le monde

Aujourd'hui, le paludisme est endémique dans de nombreux pays, principalement situés dans les zones intertropicales d'Amérique, d'Asie et bien sûr d'Afrique avec de grandes disparités régionales; l'Afrique du nord est une zone indemne de paludisme, à l'opposé de l'Afrique de l'Est, sub-saharienne et équatoriale où le paludisme est important et stable (Figure 1).

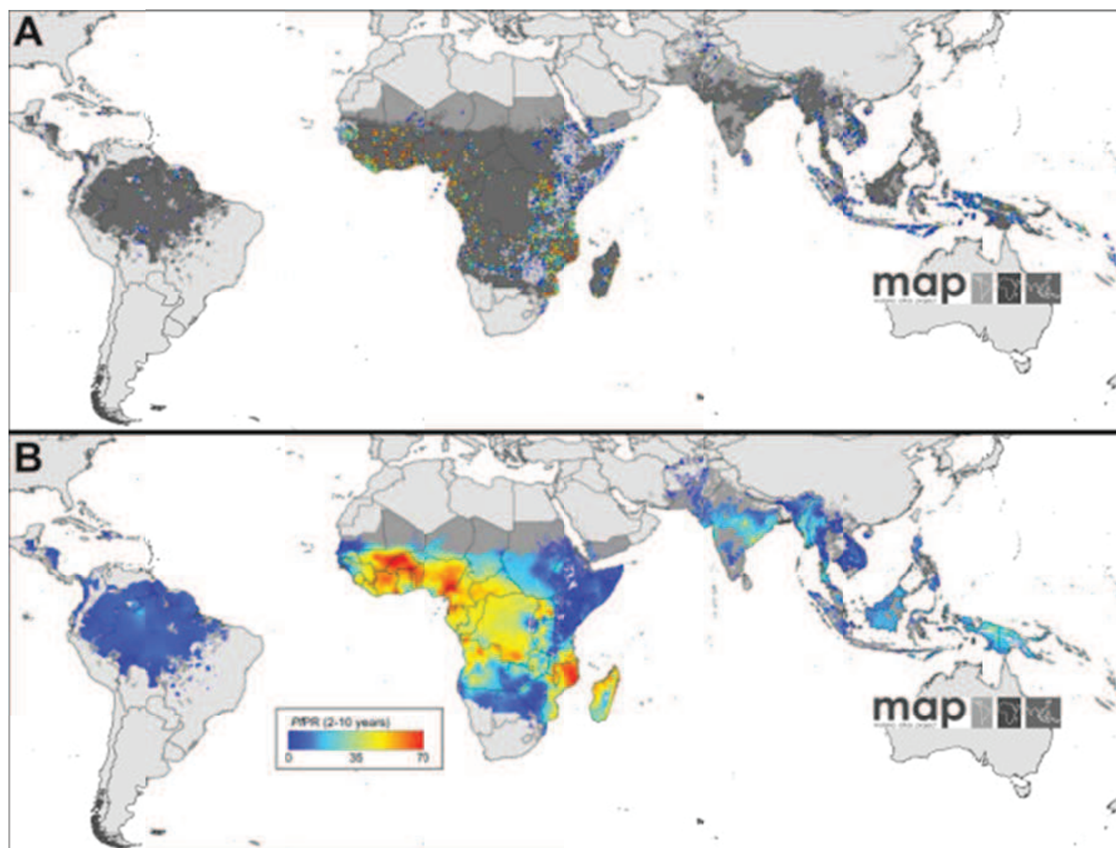

Figure 1 : Distribution spatiale de l'endémicité de Plasmodium falciparum en 2010 (Gething P. W. et al. 2011).
A. Limites spatiales du risque infectieux du paludisme à *Plasmodium falciparum* en 2010. Les régions en gris foncé sont les régions où la transmission est stable. Les autres régions du monde sont des zones où le risque de transmission du paludisme est plus faible. Cette cartographie est menée à l'échelle de 13 449 régions administratives réparties au sein de 85 pays où le paludisme est endémique. Les 22 212 mesures de cette étude sont représentées par des points de couleur variables en fonction de l'incidence parasitaire observée (mesures effectuées entre 1985 et 2010). **B.** La prévalence du paludisme chez les enfants de 2 à 10 ans (*Pf*PR$_{2\text{-}10}$) est représentée avec un code couleur (de bleu à rouge, de 0% à 100%). Cette carte a été obtenue pour l'année 2010 à l'aide d'une modélisation géostatistique (model-based geostatistics (MBG)) établi au sein des limites spatiales de transmission stable des infections chez *Plasmodium falciparum* (zones gris foncé, panel **A**).

Près de la moitié de la population mondiale (3,3 milliard de personnes) vit dans une région avec un risque de transmission palustre (zones grises, Figure 1A) et un cinquième (1,2 milliard de personnes) dans une région à fort risque (c'est-à-dire avec plus de 1 cas rapporté pour 1000 personnes par an) (WHO 2008a). L'Afrique est la région où vit le plus de personnes soumises à un risque élevé et où le nombre de cas et de décès par le paludisme est le plus élevé. Cependant, il est important de rappeler que l'évaluation précise du poids du paludisme au niveau mondial reste difficile. Les données sont souvent parcellaires avec de grandes différences d'un pays à l'autre. Par ailleurs, la mise en évidence des agents du paludisme au moyen d'un diagnostic biologique n'est pas toujours assurée et la plupart des cas répertoriés repose sur des données cliniques, dont les principaux symptômes ne sont pas spécifiques. Nous savons que les individus les plus à risque de paludisme dans les zones de forte transmission sont les individus peu immuns comme les enfants de moins de 5 ans: 75% des morts du paludisme à *P. falciparum* sont des enfants de moins de 5 ans vivant en Afrique (Snow et al. 1999).

Le paludisme à *Plasmodium vivax* (Figure 2) est surtout présent en Asie, dans certains pays d'Amérique du Sud et Centrale, en Afrique du Nord et de l'Est, et en Europe. Cette distribution est essentiellement lié au fait que *P. vivax* ne se développe pas ou moins bien chez les sujets ne possédant pas l'antigène Duffy à la surface de leur globules rouges (sujets Duffy négatifs, Fy-) (Miller et al. 1976), ce qui est le cas des populations africaines. L'antigène est peu présent dans la région afro tropicale à l'exception de certaines ethnies et la distribution de l'antigène corrèle avec la distribution de *P. vivax*. Néanmoins, ce dogme est à nuancer avec la découverte, à Madagascar, de souches de *P. vivax* capable d'envahir et d'établir son cycle chez des sujets Fy- (Menard et al. 2010). Quant aux paludismes à *Plasmodium malariae* et *Plasmodium ovale*, ils sont beaucoup moins fréquemment observés. Cependant, même à faible prévalence, *P. malariae* est présent dans de nombreux pays, alors que *P. ovale* se concentre surtout en Afrique de l'Ouest, dans la région Pacifique occidental et plus rarement en Asie. Il n'a jamais été identifié en Amérique du Sud.

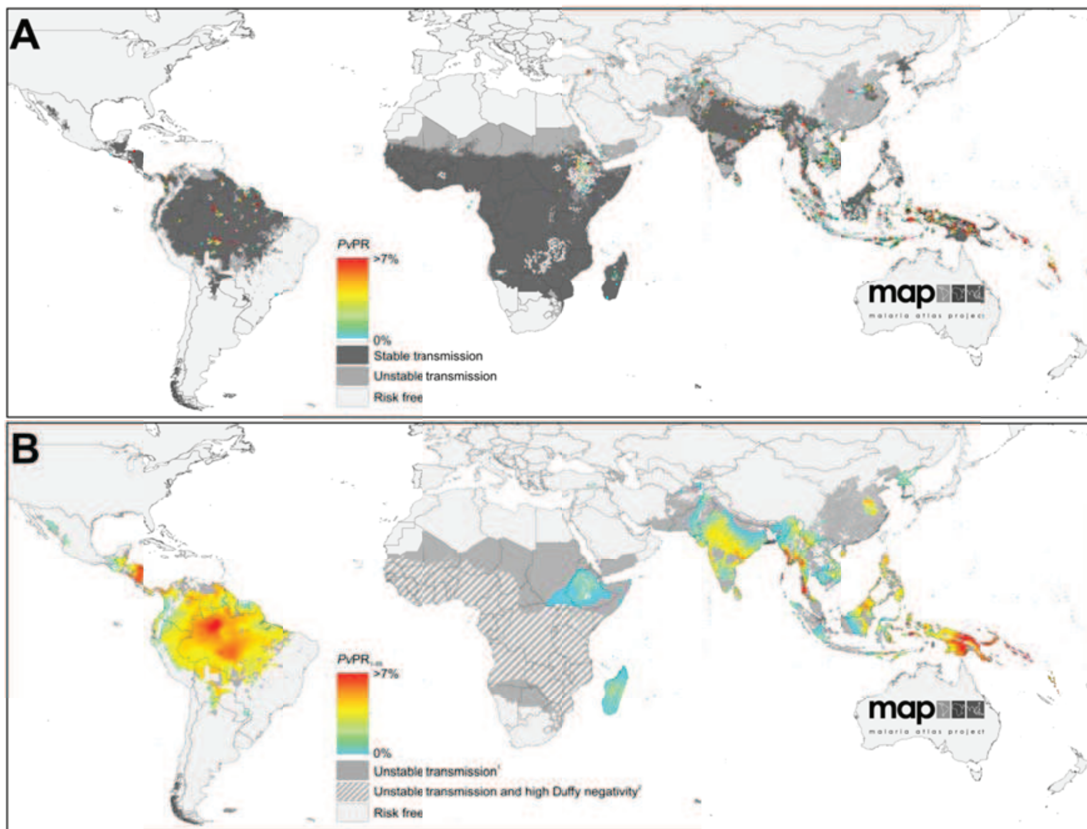

Figure 2 : Distribution spatiale de l'endémicité à Plasmodium vivax en 2010 (Gething Peter. W. et al. 2012).
A. Estimation des limites géographiques du risque infectieux lié à *Plasmodium vivax* en 2010. Les zones concernant la transmission stable, la transmission instable et l'absence de transmission sont colorées en gris foncé, gris moyen et gris clair, respectivement. Les points des couleurs variables (du vert clair au rouge, de 0% à 100%) représentent les incidences parasitaires de 9970 géolocalisations recueillis entre janvier 1985 et juin 2010. **B.** Les mêmes codes couleurs dans A sont donnés pour montrer la prévalence du *Plasmodium vivax* infectant des sujets de 1 à 99 ans ($PvPR_{1-99}$). Cette carte a été créée en se basant sur une modélisation géostatistique (model-based geostatistics (MBG)) établi au sein des limites spatiales de transmission stable des infections chez *Plasmodium vivax*. Au sein des régions de transmission stable (A), sont insérées les zones de transmission instable et les zones de forte prévalence de population Fy-.

Du faite de la réduction importante de la transmission du paludisme dans la plupart des pays endémiques (O'Meara et al. 2010, WHO 2009) un diagnostic biologique précis chez les patients suspects de paludisme est nécessaire et primordial. En effet, pour chaque cas de paludisme, un traitement spécifique et efficace doit être mis en place le plus rapidement possible (Black R. E. et al. 2010). Dans ce contexte, la mesure de l'impact des stratégies mises en place pour lutter contre le paludisme par le développement de nouveaux outils performants est une démarche essentielle. Ces outils doivent permettre en particulier de détecter les infections chez les sujets symptomatiques mais aussi chez les individus asymptomatiques (formes sexuées et asexuées) de façon à mieux évaluer l'intensité de la transmission (WHO 2008b).

2. Biologie des *Plasmodium*, diagnostics et traitements

Le paludisme est dû à des protozoaires appartenant au phylum des apicomplexes. Maladie vectorielle provoqué par la transmission d'un parasite du genre *Plasmodium.* Cette transmission intervient principalement la nuit lors de la piqûre par un moustique femelle du genre *Anophèle*s. Le cycle évolutif assez complexe nécessite donc deux hôtes : l'homme et la femelle hématophage d'un moustique du genre anophèle. D'un point de vue strictement biologique, le véritable hôte définitif de cet hématozoaire est le moustique (la reproduction sexuée parasite l'anophèle). L'homme et les autres vertébrés ne sont que des hôtes intermédiaires dans son cycle réplicatif. Les parasites, en raison de leur tropisme pour les érythrocytes, provoquent essentiellement une symptomatologie fébrile, souvent grave, parfois mortelle. Du point de vue morphologique, la cellule du parasite (stade mérozoïte) qui envahit les globules rouges est fortement polarisée. Les microtubules cytosolique irradient à partir de ce pôle apical et permettent l'organisation de plusieurs organites spécifiques de la partie antérieure du parasite : les micronèmes, les granules denses et les rhoptries (Figure 3).

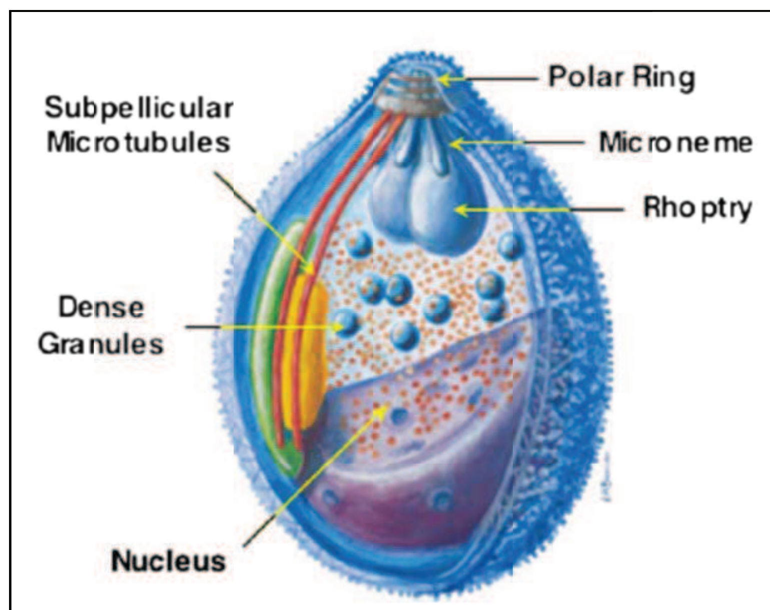

Figure 3 : Organisation cellulaire de Plasmodium (Gruner et al. 2004)
Représentation schématique des composants spécifiques des apicomplexes tels qu'ils sont observés au stade mérozoïte de *Plasmodium*. Les anneaux polaires, les micronèmes et les rhoptries sont associés à la proéminence apicale, qui tend à être marquée dans les mérozoïtes comparé aux sporozoïtes et aux ookinètes. Les rhoptries se trouvent par pair chez *Plasmodium.* Ces organelles ont une forme de poire allongée avec leurs conduits se terminant en aveugle au-dessous de la membrane plasmique. Les micronèmes sont associées à la partie étroite des rhoptries. Leur nombre et leur taille varient entre les espèces des parasites. Les granules denses sphéroïdales se trouvent dans le cytoplasme. L'arrangement des différents composants, leur nombre et leur morphologie varient entre les différences d'étapes d'invasion. Toutes ces structures et leurs contenus sont essentiels dans les mécanismes d'attachement et d'invasion de la cellule hôte.

2.1. Le cycle évolutif des parasites du paludisme

Les parasites du paludisme ont un cycle évolutif complexe. Les deux cycles connus se déroulent successivement chez l'anophèle (l'hôte définitif) et chez l'homme (l'hôte intermédiaire) (Figure 4).

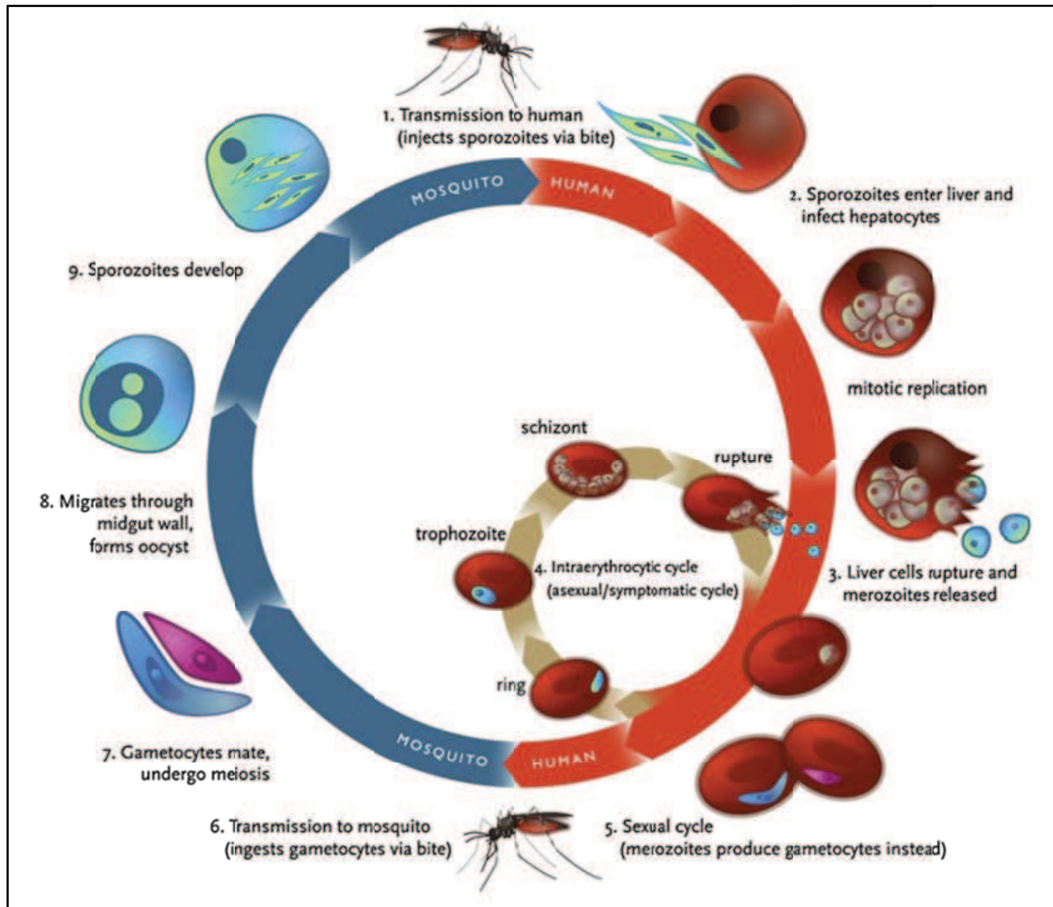

Figure 4: Schéma de cycle évolutif de Plasmodium (Klein 2013).

La transmission du paludisme se fait par un vecteur, un moustique femelle, du genre *Anophèle*, qui ingère des gamétocytes mâles et femelles lors de repas du sanguin chez l'homme infecté. Au sein de son estomac, les gamétocytes se différencient en gamètes et fusionnent lors de la fécondation. L'ookinète ainsi formé traverse la paroi de l'estomac de moustique et se différencie alors en oocyste. La méiose conduit à la formation des sporoblastes. Ceux-ci vont générer des milliers de sporozoïtes qui migreront vers les glandes salivaires. Ils sont ensuite injectés chez l'homme lors d'un nouveau repas du sang, puis atteignent le foie et infectent les hépatocytes où ils commencent une réplication asexuée mitotique. Environ 6-15 jours plus tard, des milliers de mérozoïtes quittent les cellules hépatiques et envahissent les globules rouges dans la circulation sanguine. Ce nouveau cycle érythrocytaire dure 48h. Les parasites progressent dans le cycle en passant à travers différents stades: stade d'anneau, de trophozoïte, de schizonte. Un schizonte conduit à la formation de 16 à 32 mérozoïtes. Ces globules rouges infecté libèrent de façon synchrone les nouveaux parasites et provoquent un cycle fébrile caractéristique des signes cliniques de la maladie. Après chaque division, une partie des mérozoïtes se différencient en gamétocytes qui peuvent infester des moustiques sensibles, ce qui permet au cycle de transmission de recommencer.

2.1.1. Chez le vecteur

Au cours d'un repas sanguin sur un individu infecté, l'anophèle femelle ingère les différentes formes du parasite (étape 6-9, Figure 4). Les gamétocytes, formes sexuées mâles et femelles vont poursuivre leur développement. Sous l'effet des nouvelles conditions environnementales, les gamétocytes femelles se transforment en microgamètes immobiles, les gamétocytes mâles « exflagellent » et se transforment en microgamètes. La fécondation du macrogamète et du microgamète forme un zygote diploïde devenant mobile: l'ookinète. Il traverse la paroi stomacale et forme alors l'oocyste (Sinden R. E. and Billingsley 2001). Il subit alors une méiose formant des masses haploïdes appelées sporoblastes. Ceux-ci vont se diviser et générer des milliers de sporozoïtes qui migreront vers les glandes salivaires (Beier 1998). Cette étape se déroule en 24 heures environ.

2.1.2. Chez l'homme

Chez l'homme, le cycle biologique des *Plasmodium* est diphasique avec une phase hépatique ou pré-érythrocytaire (= exo-érythrocytaire), qui correspond à la phase d'incubation, cliniquement asymptomatique et une phase sanguine ou érythrocytaire qui correspond à la phase clinique de la maladie.

Phase hépatique ou pré-érythrocytaire

Lors d'un repas sanguin (étape 1, Figure 4), l'anophèle infectée injecte les sporozoïtes présents dans ses glandes salivaires. Ces cellules infectantes vont rapidement (moins d'une demi-heure) circuler dans le sang et atteindre le foie (étape 2, Figure 4). Cette phase permet ensuite aux sporozoïtes d'infecter les hépatocytes (Sinden R.E. 1998). Cette crise hépatique va durer de 7 à 15 jours pour *P. falciparum*, de 15 jours à 9 mois pour *P. vivax*, de 15 jours à 10 mois pour *P. ovale* et 3 semaines pour *P. malariae*. Les sporozoïtes qui n'atteindront pas le foie, sont éliminés par les cellules phagocytaires. A ce stade, *Plasmodium* est incapable de poursuivre son cycle dans les cellules d'autres organes.

Le trophozoïte est une cellule uninucléée de grande taille. Le parasite va s'engager dans une série de mitoses successives par schizogonie aboutissant à une cellule polynucléée : le schizonte hépatique. Le schizonte est aisément reconnaissable car il est riche en acides nucléiques et donne une coloration bleu pâle aux cellules hépatiques colorées au May-Grünwald-Giemsa. Au bout d'une semaine à quinze jours, le schizonte se segmente en mérozoïtes. Les jeunes mérozoïtes sont libérés dans le sang après rupture de la membrane plasmique de la cellule hôte (étape 3, Figure 4), initiant ainsi le stade érythrocytaire, c'est-à-dire l'infection des globules rouges.

Certains mérozoïtes de *P. ovale* (Cogswell 1992) ou *P. vivax* (Krotoski et al. 1982) peuvent rester inactifs dans le foie pendant plusieurs années (Mouchet et al. 2004). Le parasite reste en « phase dormante » : le *Plasmodium* n'évolue pas mais dort, d'où le nom qui lui est donné d'« hypnozoïte ». Les hypnozoïtes sont caractéristiques de *P. vivax* et *P. ovale*. Les parasites peuvent rester dans le foie 2 ou 3 ans pour *P. ovale*, 3 à

5 ans ou plus pour *P. vivax* selon l'origine des souches (souches d'origine tropicale ou d'origine tempérée). Il n'y a pas de forme hypnozoïte décrite chez *P. falciparum* et chez *P. malariae* (Greenwood et al. 2008, Tanomsing et al. 2007). Les mérozoïtes sont libérées dans les sinusoïdes hépatiques (vaisseaux capillaires du foie faisant la jonction entre celui-ci et le réseau sanguin) par rupture de l'enveloppe cellulaire (Mouchet et al. 2004). Les jeunes mérozoïtes « pré-érythrocytaires » se répandent dans la circulation sanguine pour infecter les globules rouges.

Phase érythrocytaire

Cette phase est responsable des symptômes. Il s'agit d'une phase de prolifération asexuée qui se déroule dans les érythrocytes (étape 4, Figure 4). Le mérozoïte pénètre grâce à un processus parasitaire actif et se différencie au sein de la vacuole parasitophore en anneau, puis en trophozoïte. L'ensemble de ce cycle dure 48 heures chez *P. falciparum*.

Stade anneau. Ce nom est associé à son apparence caractéristique en « bague à chaton » sur frottis sanguin, coloré au Giemsa (*P. falciparum*). Cela est dû au fait que tous les organites majeurs (un noyau, des mitochondries, un apicoplaste, un réticulum endoplasmique, des ribosomes) sont en périphérie, et que le centre du disque, très fin, ne contient plus que le cytosol. A ce stade, le parasite commence à se nourrir, en dégradant entre autre l'hémoglobine de la cellule hôte. Il va se transformer en une forme plus arrondie: le trophozoïte.

Stade trophozoïte. Morphologiquement, le stade trophozoïte se différencie du stade anneau essentiellement par la taille. L'activité métabolique du parasite s'accroit fortement. Le volume du cytoplasme et la taille des mitochondries et de l'apicoplaste augmentent. Le parasite continue à se nourrir en dégradant l'hémoglobine. Il transforme l'hème, toxique, en pigment inerte l'hémozoïne qu'il accumule dans la vacuole digestive. Le parasite modifie progressivement la membrane des globules rouges en exportant différents protéines. L'adressage des protéines parasitaires à la surface du globule rouge infecté est favorisé par la formation d'un réseau tubule-vésiculaire (TVN). Le TVN se forme à partir de la membrane parasitophore. Les « tâches de Maurer » forment un compartiment essentiel du TVN impliqué dans la maturation des protéines adressage à la membrane plasmique de l'érythrocyte (Tilley et al. 2008, Wickham et al. 2001).

Stade schizonte. Il s'agit d'un stade de divisions nucléaires répétitives, suivies de divisions cytoplasmiques. La digestion de l'hémoglobine se poursuit jusqu'à la fin de ce stade et l'hémozoïne s'agglomère en une masse dense. Au cours de la biogénèse des mérozoïtes, l'acquisition des organites spécifiques de l'embranchement des apicomplexes (rhoptries, micronèmes et granules denses) s'effectue. En l'absence de traitement, tous les parasites évoluent progressivement au même rythme dans les globules rouges (on dit qu'ils sont synchrones). Les schizontes érythrocytaires arrivent à maturation au même moment et la libération des mérozoïte sera simultanée. La destruction d'un grand nombre de globules rouges de manière périodique, toutes les 24 heures pour *P. knowlesi*, toutes les 48 heures pour *P. falciparum, P. vivax* ou *P.*

ovale ou toutes les 72 heures pour *P. malariae* entraine une réponse de l'organisme qui est caractéristique de la maladie et de chaque parasite (fièvre tierce de *P. falciparum, P. vivax* ou *P. ovale* et fièvre quarte de *P. malariae*). En pratique, la fièvre tierce chez *P. falciparum* est rarement synchrone. L'éclatement des schizontes mûrs termine le premier cycle schizogonique érythrocytaire en libérant dans le sang les mérozoïtes « érythrocytaires » capables d'infecter de nouveaux globules rouges. Après un certain nombre de cycles érythrocytaires, certains mérozoïtes subissent une maturation d'une dizaine de jours, accompagnée d'une différenciation sexuée: ils se transforment en gamétocytes mâles et femelles.

Le paludisme peut évoluer vers des formes plus grave "neuropaludisme" (accès pernicieux = « cerebral malaria » pour les anglo-saxons) qui est la complication majeure du paludisme à *P. falciparum.*

2.2. Les infections mixtes

Une infection mixte correspond à la présence chez un même individu, en phase intra érythrocytaire, d'au moins deux espèces plasmodiales différentes. Il a été mis en évidence que les infections mixtes avec *P. falciparum* et *P. vivax* entraînaient une variabilité de la densité parasitaire de chacune des espèces (Boyd and Kitchen 1937, 1938). Ainsi, la plupart du temps, l'examen microscopique d'un individu atteint d'une infection mixte, une seule espèce est détectée à la fois. Il y avait succession d'épisodes de parasitémie soit à *P. falciparum*, soit à *P. vivax*. Lors d'une étude épidémiologique (Cohen 1973), la prévalence des infections mixtes était moins élevés qu'attendue. Cette observation a été suivie par d'autres qui ont permis de préciser les conséquences ces infections chez l'homme. D'autres études menées en Côte d'Ivoire et au Sri Lanka, publiées en 1994, ont montré une diminution significative de la symptomatologie des infections chez *P. falciparum* lorsque celle-ci était associée à une infection à *P. vivax* et/ou *P. malariae* (Black J. et al. 1994, Gunewardena et al. 1994). Une diminution de l'intensité des fièvres a également été constatées dans le cas d'une infection mixte à *P. falciparum* et *P. malariae* (Black J. et al. 1994). Plus récemment, une étude réalisée en Thaïlande a montré que des patients présentant une infection mixte à *P. falciparum* et à *P. vivax* développaient moins souvent de formes graves dues à *P. falciparum* (risque de développer une forme grave lors d'une monoinfection à *P. falciparum* de 5,7 % *vs.* infection mixte à *P. falciparum* et *P. vivax* de 1,6 %) (Luxemburger et al. 1997). Ces résultats ont été confirmés ultérieurement chez les enfants africains, où l'infection mixte à *P. falciparum* et à *P. malariae* et/ou *P. ovale* engendrait moins de symptômes palustres graves par rapport aux enfants présentant une infection simple (Alifrangis et al. 1999). Une explication de cette interaction apparente entre les infections à *P. falciparum* et à *P. vivax* serait l'existence d'une réponse immunitaire croisée, dirigée contre les deux espèces (Maitland et al. 1997). Il a également été proposé que l'infection par une espèce pourrait aussi diminuer la capacité de transmission d'une autre espèce. Des études menées au Vanuatu ont montré une complémentarité saisonnière des infections à *P. falciparum* et à *P. vivax.* C'est-à-dire qu'en fonction de la saison, une espèce était retrouvée de manière majoritaire (Maitland et al. 1997, Molineaux et al. 1980). Cela pourrait être dû tout autant à une diminution réelle de transmission d'une

espèce à cause de la présence de l'autre qu'à une diminution de sensibilité de détection microscopique d'une espèce minoritaire dans le cas d'une coinfection.

Par ailleurs, l'ensemble des études épidémiologiques des infections mixtes a longtemps reposé sur le diagnostic microscopique des infections. Les techniques de biologie moléculaire ont permis de diminuer le seuil de détection des parasites et d'augmenter la spécificité du diagnostic d'espèce (Brown et al. 1992). Des études utilisant ces techniques ont révélé que le nombre d'infections mixtes étaient largement sous-évaluées. Leur proportion a pu être augmentée de 55 à 65 % en utilisant des méthodes moléculaires plus sensibles par rapport à la microscopie (Mayxay et al. 2004). Les conclusions de l'ensemble des résultats épidémiologiques portant sur les infections mixtes devraient donc être réexaminées à l'aide des nouvelles technologies (Paul et al. 1999).

2.3. Diagnostic du paludisme

Le diagnostic du paludisme est un problème préoccupant pour la prise en charge efficace de cette maladie. En zone d'endémie palustre, le diagnostic repose souvent sur le seul diagnostic de fièvre. Il n'y a pas de symptôme pathognomonique du paludisme (Chandramohan et al. 2002). Inversement, la présence du parasite dans le sang ne suffit pas à lui seul à expliquer les symptômes et les signes cliniques du paludisme.

2.3.1. Diagnostic clinique

Les signes accompagnant un accès palustre simple à *Plasmodium falciparum* sont très variables et peuvent se confondre avec d'autres maladies telles que la grippe, les hépatites, la typhoïde, une méningite, une gastro-entérite etc.. Le malade se plaint fréquemment de fièvre, de maux de tête, de douleurs divers (quelque fois raideur de la nuque), de douleurs abdominales et de diarrhées. Le jeune enfant peut être irritable, peut refuser de s'alimenter et vomir. La fièvre peut malgré tout être absente. Elle est tout d'abord continue plutôt tierce (pics de fièvre un jour sur deux) et elle peut ou non s'accompagner de frissons. Un paludisme à *Plasmodium falciparum*, qui n'est pas traitée rapidement, peut évoluer vers un paludisme grave, souvent mortel.

2.3.2. Diagnostic biologique

Un diagnostic adéquat est important pour assurer que le traitement donné est efficace (traitement antipaludique ou non si diagnostic négatif). Dans la plupart des régions d'Afrique, le diagnostic du paludisme est fondé sur la symptomatologie. Or, le diagnostic clinique du paludisme est très imprécis, étant donné que les symptômes sont aspécifiques et peuvent être la manifestation d'autres maladies infectieuses fébriles. Le diagnostic précis du paludisme doit reposer sur des tests biologiques. Le diagnostic biologique peut s'effectuer par différentes techniques.

Détection microscopique

La détection microscopique des parasites est considérée comme le diagnostic de référence du paludisme. Les parasites sont généralement colorés au Giemsa qui colore le cytoplasme des globules rouges en rose, et les noyaux de *Plasmodium* en violet. La goutte épaisse permet de détecter une parasitémie faible (de l'ordre de 10 parasites/µl) alors que l'examen d'un frottis mince qui est moins sensible (100 parasites/µl) est réservé à l'identification de l'espèce (Ndao 2009).

Détection des antigènes palustres par tests de diagnostic rapide (TDR ou RDT)

De nouvelles techniques de diagnostic rapide ont été développées (Murray and Bennett 2009) à la fin des années 90. Il s'agit notamment de tests immunochromatographiques. Ces tests sont basés sur le principe de la détection de 3 protéines parasitaires : la protéine riche en histidine 2 (HRP-2), la lactate déshydrogénase (pLDH) ou l'aldolase (Banoo et al. 2006, Bell et al. 2006).
La protéine HRP-2 est une protéine soluble spécifique de *Plasmodium falciparum*. Elle est localisée dans le cytoplasme et sur la surface membranaire de l'érythrocyte infectée. Cette protéine est essentiellement retrouvée aux stades asexués, mais également présente chez les gamétocytes jeunes chez *Plasmodium falciparum.* Abondant, cette protéine peut être détectée jusqu'aux 28 jours après la prise d'un traitement efficace (Karbwang et al. 1996, Richter et al. 2004, Swarthout et al. 2007). La protéine pLDH, est une enzyme glycolytique des *Plasmodium.* Elle est utilisée pour détecter les parasites aux stades asexués et sexués. Les anticorps monoclonaux dirigés contre la pLDH peuvent cibler toutes les espèces du paludisme infectant l'homme mais peut être également être spécifique d'espèce (*P. falciparum* ou *P. vivax)* (Makler et al. 1998). Enfin, l'aldolase est une autre enzyme qui peut être utilisé pour détecter le genre *Plasmodium*, sans différenciation des espèces (Genrich et al. 2007, Lee et al. 2006).

Quantitative Buffer Coat (QBC)

Le QBC, distribué par la société Becton-Dickinson, est basé sur la détection des globules rouges infectés par coloration de l'ADN parasitaire à l'acridine orange. Après centrifugation au sein d'un tube capillaire, les éléments du sang sont séparés en fonction de leur densité. Les globules rouges parasités occupent la phase supérieure de la colonne. La présence de parasite et le nombre de parasite sont alors évalués au microscope à fluorescence. La sensibilité de cette technique est proche de celle de la goutte épaisse. Elle permet de distinguer les différentes espèces mais nécessite un appareil ainsi que des réactifs coûteux et demande une certaine expérience (Plowe et al. 1997).

Détection des acides nucléiques parasitaires par les techniques d'amplification génique

La mise au point de la technique d'amplification génétique (Polymerase Chain Reaction, PCR) a permis de mettre au point des techniques permettant la détection du génome parasitaire, en présence de l'ADN humain par une amplification d'un gène ciblé. Les méthodes les plus utilisées sont la PCR classique (conventional PCR) ou la PCR en temps réel (real time-PCR). Les premières PCR ont été mise au point en 1990

(Jaureguiberry et al. 1990). L'identification de *Plasmodium spp.* utilisant deux PCR nichées (nested PCR) a été initialement décrite par Snounou et al. (Snounou et al. 1993), puis améliorée par Singh et al. (Singh et al. 1999) en ciblant le gène de la sous unité *18S* du ribosome. Elle reste couramment utilisée et est souvent considéré comme la méthode de référence. La PCR peut être également utilisée pour distinguer différents génotypes parasitaires. Cette méthode est utile pour savoir si le génotype initial est responsable de la recrudescence constatée après un traitement antipaludique (distinction entre une recrudescence et une réinfection dans l'étude des échecs thérapeutiques). Les gènes classiquement ciblés sont les protéines de surface des mérozoïtes (*msp1, msp2*) ou la protéine riche en glutamate (*glurp*) (Ayala et al. 2006, Cattamanchi et al. 2003, Snounou et al. 1999). La PCR est également utilisé pour détecter des mutations au sein de gènes associés avec la résistance des parasites aux antipaludiques, ce que nous verrons plus en détails dans le chapitre suivant.

2.4. Mécanismes d'action des antipaludiques et mécanismes de résistance des parasites

Limitées essentiellement au stade intra-érythrocytaire, les études biochimiques chez *Plasmodium* ont permis de mieux comprendre les voies métaboliques mises en place par le parasite et ainsi développer des nouveaux antipaludiques agissant sur ces voies.
Les antipaludiques actuels peuvent être classés selon leur mode d'action comme présenté dans la Figure 5. Par exemple, la vacuole nutritive du parasite est le siège de la digestion de l'hémoglobine et de la cristallisation de l'hème sous forme d'hémozoïne. Les amino-4-quinoléines (chloroquine, amodiaquine) et les amino-alcools (quinine, méfloquine, halofantrine, luméfantrine) interviennent dans le métabolisme de l'hémoglobine situé au niveau de la vacuole nutritive en inhibant la formation de l'hémozoïne. De plus, le cytoplasme contient le cytosol et les deux organites importants, la mitochondrie et l'apicoplaste qui interviennent dans la biosynthèse des acides nucléiques. Les antifolates, incluant les antifoliniques (sulfadoxine, sulfone) et les antifoliques (proguanil et pyriméthamine), agissent au niveau de voie de synthèse de folates. Ils jouent un rôle très important dans la biosynthèse des acides nucléiques. Les antifoliques inhibent la dihydroptéroate synthétase (DHPS) qui produit l'acide folique, les antifoliniques inhibent la dihydrofolate réductase (DHFR) qui produit l'acide folinique. Tandis que la membrane plasmique qui comporte des phospholipides, des canaux calciques et parasitophore, est la place de circulation nutritionnelle.
http://medecinetropicale.free.fr/spe/paluaubry.htm

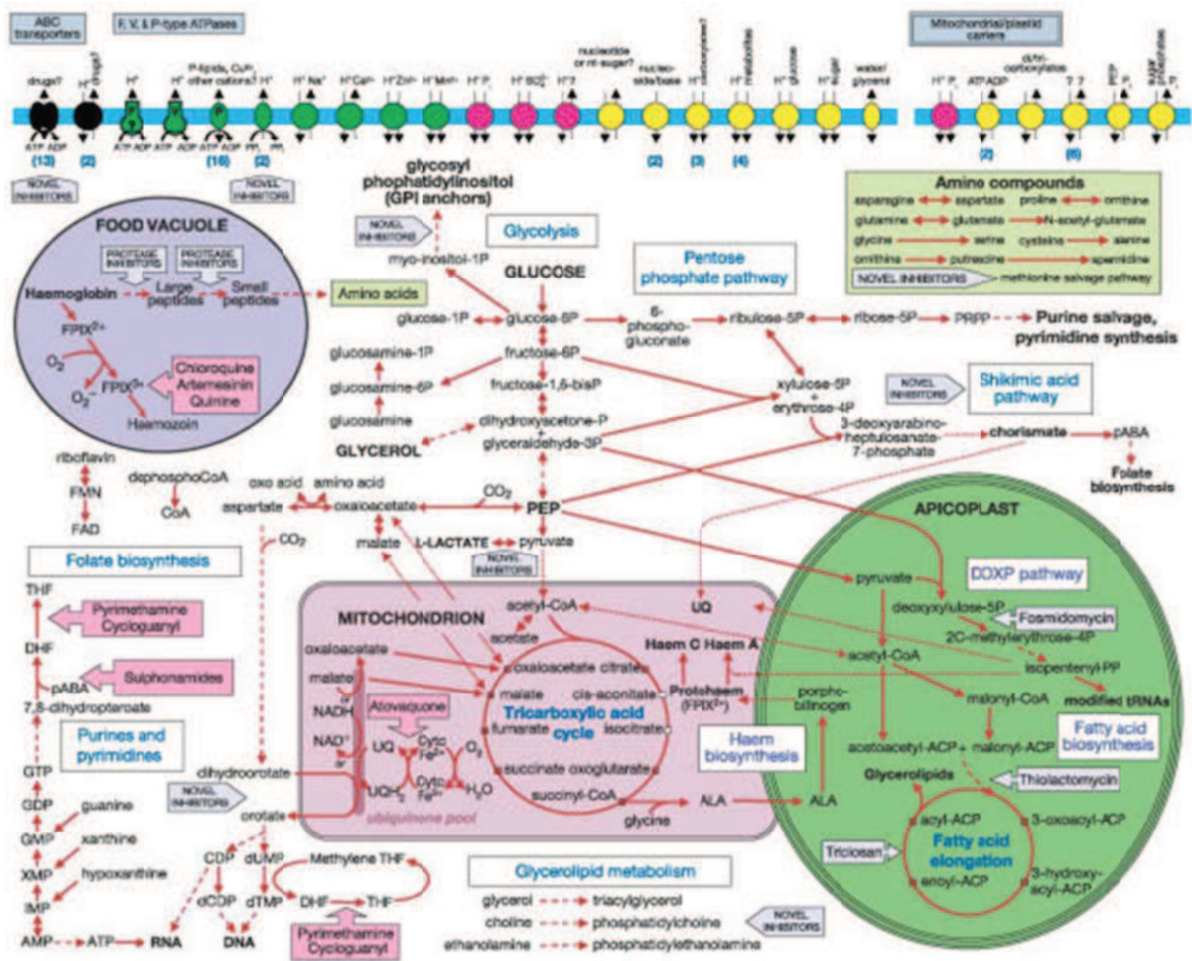

Figure 5. Métabolismes et transport chez Plasmodium falciparum (Carlton et al. 2002)

Le glucose et le glycérol sont les principales sources de carbones pour *Plasmodium*. Les étapes métaboliques sont indiquées par les flèches, les lignes pointillées montrent les multiples étapes d'interaction, les flèches pointillées indiquent les voies incomplètes, inconnues ou douteuses. La localisation des organites connus ou potentiels est représentée par les voies associées à la vacuole digestive, à la mitochondrie et à l'apicoplaste. Les petits carrés blancs indiquent les cycles métaboliques TCA (acides tricarboxyliques) qui peuvent provenir de l'extérieur de la mitochondrie. Les blocks avec flèches fuchsia montrent les stades d'action des antipaludiques ; les blocks avec les flèches grises mentionnent les cibles potentielles aux médicaments. Les transporteurs sont groupés par les substrats spécifiques : les cations inorganiques (vert), les anions inorganiques (magenta), les nutriments organiques (jaune), les efflux médicamenteux et autres (noir). Les flèches indiquent la direction du transport des substrats et des ions. Les chiffres indiqués en parenthèse représentent les gènes de transports des substrats. La localisation des transporteurs membranaires inconnus ou subcellulaires sont montrées dans la membrane (barre bleue). Abreviations: ACP, acyl carrier protein; ALA, aminolevulinic acid; CoA, coenzyme A; DHF, dihydrofolate; DOXP, deoxyxylulose phosphate; FPIX2 et FPIX3., ferro- and ferriprotoporphyrin IX, pABA, para-aminobenzoic acid; PEP, phosphoenolpyruvate; Pi, phosphate; PPi, pyrophosphate; PRPP, phosphoribosyl pyrophosphate; THF, tetrahydrofolate; UQ, ubiquinone.

Comme tout microorganisme, les *Plasmodium,* pour survivre, mettent en place des mécanismes de résistance pour contrer les effets des antipaludiques. Cette adaptation et cette plasticité moléculaire semblent

être de plus en plus rapides et efficaces comme le montre la figure 6 (délai entre l'introduction des antipaludiques et premiers cas cliniques de résistance observés).

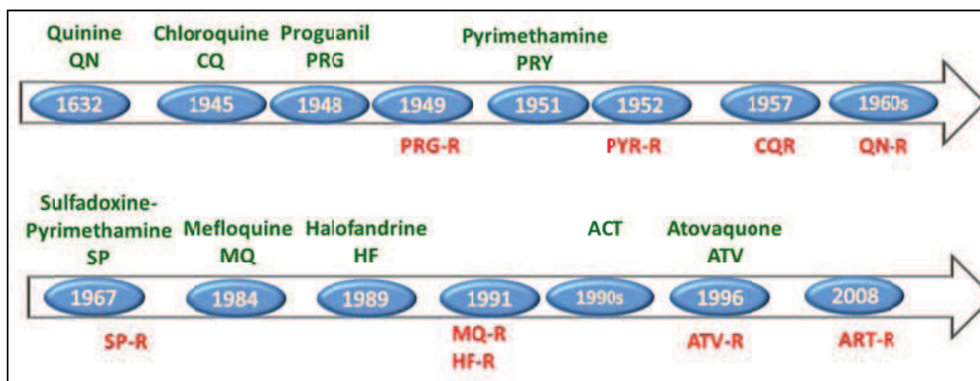

Figure 6 : Délai entre l'introduction des antipaludiques et premiers cas cliniques de résistance observés
(exemple : pour la pyrimethamine, introduction en 1951 dans le traitement du paludisme non compliqué à *P. falciparum* et premier cas de résistance observé en 1952).

2.4.1. Quinine (QN)

La quinine (QN), extraite de l'écorce de quinquina (*cinchona*) en 1632, a été le premier antipaludique utilisé (Toovey 2004). Son extraction a ensuite été mise au point en 1817 par deux chercheurs Français, Pierre Joseph Pelletier et Joseph Bienaimé Caventou. La QN, un dérivé de quinoléine, est une base monoprotique faible qui s'accumule au sein d'un environnement à pH faible comme la vacuole digestive de *Plasmodium falciparum*. La quinine s'associe à l'hème en inhibant la biocristallisation de l'hémozoïne ce qui facilite d'agrégation des cytotoxiques de l'hème (Dorn et al. 1998). La QN est couramment recommandée pour le traitement des cas sévères du paludisme, des femmes enceintes infectées ou dans le traitement de deuxième ligne en association avec des antibiotiques (WHO 2010). En Asie du Sud-Est, la QN est utilisée en association avec la tétracycline (Duarte et al. 1996, Looareesuwan et al. 1992) ou la clindamycine (Kremsner 1990).

Les premiers cas résistants à la QN documentés ont été rapportés dans les années 1960 au Brésil et en Asie du Sud-Est (Bjorkman and Phillips-Howard 1990, Giboda and Denis 1988). Les cas de résistance sont devenus ensuite sporadique depuis les années 1980 (Harinasuta et al. 1990, Jelinek et al. 1995, Tish and Pillans 1997).

En 2004, l'analyse par QTL (Quantitative Trait Loci) de croisements génétiques des clones HB3 et Dd2, (Ferdig et al. 2004) a permis d'identifier que les gènes *Pfmdr-1* (*Pf multidrug resistance 1*) sur le chromosome 5, *Pfcrt* (*Pf chloroquine resistance transporter)* sur le chromosome 7, et *pfnhe-1* (*P. falciparum Na+/H+ exchanger-1*) sur le chromosome 13 étaient associés à une sensibilité réduite à la QN. Cette étude a

permis de confirmer les résultats obtenus à partir de souches de terrain et en particulier de confirmer l'implication des gènes *Pfcrt* (Sidhu et al. 2006) et *Pfmdr-1* (Reed et al. 2000). L'introduction expérimentale de mutations au sein du gène *Pfmdr-1* a été associée à une augmentation *in vitro* de la résistance à la QN (Mu et al. 2003). La surexpression *in vitro* du gène *Pfmdr-1* a permis de montrer une résistance croisées entre la QN et la méfloquine (MF) (Cowman et al. 1994, Peel et al. 1994). Il a été également observé que les isolats résistants à la MQ étaient le plus souvent sensibles à la QN et vice versa (Ang et al. 1997).

La QN reste toujours efficace contre les souches résistantes à la chloroquine (CQ). Ainsi, les phénotypes de réponse à la QN sont sans doute liés à d'autres gènes. Des études épidémiologiques récentes montrent que les polymorphismes d'un microsatellite flanquant le gène *pfnhe-1* (*ms4760*) peuvent être associé à la résistance à la QN (Ferdig et al. 2004, Henry et al. 2009), mais les données sont conflictuelles.

2.4.2. Chloroquine (CQ)

La chloroquine (CQ) a été découverte en 1934 (Krafts et al. 2012) et a été largement utilisée comme médicament de choix dans le traitement du paludisme après la deuxième guerre mondiale.

Les premières souches de *P. falciparum* résistantes à la CQ sont apparues successivement dans 4 régions distinctes (Figure 7) (Mita et al. 2009). La première région atteinte a été l'Asie du Sud-Est, le long de la frontière Khmero-Thaillandaise (Eyles et al. 1963, Harinasuta et al. 1965). Les premiers cas de résistance ont été décrits en 1957, puis la résistance s'est rapidement étendue vers la Thaïlande. Deux autres régions ont été identifiées en 1960 en Amérique du Sud : au Vénézuela et en Colombie (Moore and Lanier 1961). En 1976, 2 cas de chloroquino-résistance ont été rapportés de Papouasie Nouvelle-Guinée à Port Moresby (Grimmond et al. 1976), représentant le quatrième foyer d'émergence. Une étude récente portant sur la génétique des populations a permis de mettre en évidence que, malgré leur relative proximité géographique, les souches de Papouasie Nouvelle-Guinée étaient bien différentes de celles d'Asie du Sud-Est (Mehlotra et al. 2001). En 1978, les premières souches africaines chloroquino-résistantes ont été découvertes chez des sujets non immuns au Kenya et en Tanzanie (Campbell et al. 1979, Fogh et al. 1979), puis quelques années plus tard à Madagascar (Aronsson et al. 1981). La chloroquino-résistance s'est ensuite étendue de la côte orientale de l'Afrique et a été observée en 1983 au Soudan, en Ouganda (Onori 1984), en Zambie (Ekue et al. 1983) et au Malawi (Fogh et al. 1984, Overbosch et al. 1984, Slatter et al. 1983), laissant penser que les souches chloroquino-résistance avaient diffusé de l'Asie du Sud-Est vers l'Afrique en raison des mouvements de populations. Cette hypothèse a été clairement confirmée en montrant la similarité entre les souches asiatiques et africaines et leurs différences avec les souches d'Amérique du Sud et de Papouasie Nouvelle-Guinée (Wellems and Plowe 2001). Des résultats de transfection (Fidock et al. 2000) ainsi que de nombreuses études établissant une association entre le génotypage d'isolats et leur phénotype (Basco and Ringwald 2001, Chen et al. 2001, Fidock et al. 2000) ont permis de confirmer l'implication directe du gène *Pfcrt* (*P. falciparum chloroquine resistant transporter*) (chromosome 7). Il existe plusieurs hypothèses

concernant la fonction de *Pfcrt*, protéine de transport au niveau de la membrane de la vacuole digestive : *Pfcrt* muté pourrait expulser activement la CQ hors de la vacuole digestive (Nessler et al. 2004), altérer le pH de la vacuole digestive et permettre ainsi à la CQ diprotonée d'être transportée passivement vers le cytosol (Johnson et al. 2004).

La majorité des pays en Afrique, la CQ a été utilisée jusqu'en 1988. Elle a ensuite été remplacée par l'association sulfadoxine-pyrimethamine (SP) (Bredenkamp et al. 2001).

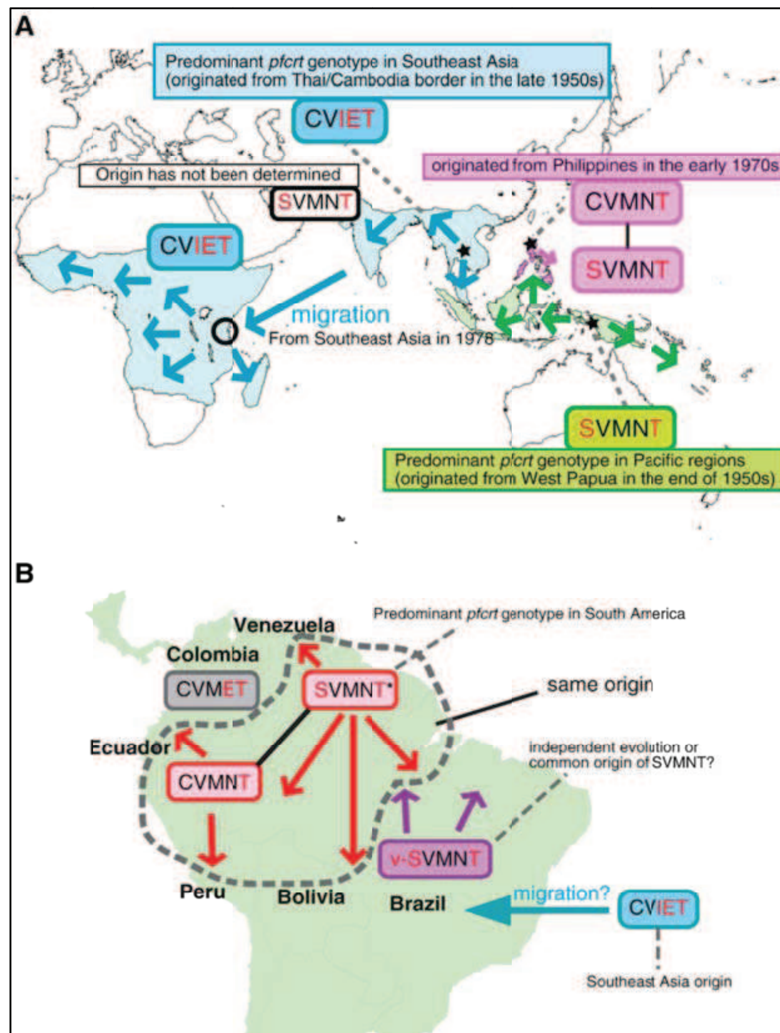

Figure 7: Origine et diffusion géographique de la résistance à la chloroquine (Mita et al. 2009).
A : Diffusion de la resistance à la chloroquine (CQR) en Asie, Afrique et dans la région Pacifique. Trois origines indépendantes des souches CQR ont été découvertes en Asie du Sud-Est, en Papouasie Occidentale et aux Philippines. Les haplotypes du gène *Pfcrt* CV**IET**, **S**VMN**T** et CVMN**T** ont été observés. La migration de l'haplotype CV**IET** de l'Asie du Sud-Est en Afrique est une voie très remarquable de la diffusion de la résistance à la chloroquine.
B. Diffusion de la résistance à la chloroquine (CQR) en Amérique du Sud. L'haplotype **S**VMN**T** du gène *Pfcrt,* originaire de Venezuela, apparu à la fin des années 1950s, est maintenant largement distribué dans tout le continent. L'haplotype CVMN**T** observé au Pérou, en Equateur et au Brésil a probablement évolué à partir de l'haplotype **S**VMN**T**. L'haplotype CVM**ET** pourrait provenir de Colombie à la fin des années 1950s et n'est limité qu'à Colombie.

2.4.3. Sulfadoxine-pyrimethamine (SP)

La pyrimethamine est un médicament de la famille des antifolates. La pyriméthamine interfère avec la synthèse d'acide tetrahydrofolate à partir d'acide folique en inhibant l'enzyme dihydrofolate reductase (DHFR). L'acide tetrahydrofolique est nécessaire à la synthèse d'ADN ou ARN dans nombreuses espèces incluant les protozoaires. La résistance aux antifolates, contrairement à celle de la CQ, est apparue presque immédiatement après leur utilisation et indépendamment dans plusieurs régions (Figure 8). Les premiers traitements contre *Plasmodium falciparum* avec la pyrimethamine ont été réalisés chez des enfants en Afrique, en 1951 (Archibald 1951). A ce moment-là, la chloroquine était efficace et la pyrimethamine a été utilisée en prophylaxie, en traitement de masse. La résistance à la pyrimethamine est apparue rapidement après son introduction (Burgess and Young 1959, Clyde and Shute 1954, Jones 1954, Meuwissen 1961). C'est pourquoi elle a été par la suite utilisée en combinaisons avec les sulfamides (Hyde 2007). Les sulfamides, comprennent la sulfadoxine, la sulfalène et le sulfone. Les sulfamides inhibent les activités de la dihydropteroate synthase (DHPS), enzyme unique intervenant dans la biosynthèse de coenzyme de l'acide folique des parasites. La combinaison Sulfadoxine-Pyrimethamine (SP) est hautement active contre les schizontes sanguins de *P. falciparum*. Le Malawi a été le premier pays africain à inscrire la SP à la place de la CQ dans sa politique nationale de lutte contre le paludisme, suivi 4 ans plus tard par le Kenya, les autres provinces d'Afrique du Sud et le Botswana (Bloland et al. 1993, Bloland et al. 1998). La SP a été introduite en 1967 en Thaïlande et la résistance à ce médicament rapportée la même année (Peters 1987, Wongsrichanalai et al. 2002).

Le rapide délai d'apparition des souches résistantes est probablement lié au type de mutations nécessaires (Fidock et al. 2000, Foote and Cowman 1994, Wellems et al. 1990). La résistance est causée par des mutations ponctuelles dans les gènes de la *dihydrofolate reductase* (DHFR) et de la *dihydropteroate synthase* (DHPS). Les changements des acides aminés en positions 51, 59, 108 et 164 dans le gène DHFR sont fortement associés à l'échec thérapeutique à la pyriméthamine chez *Plasmodium falciparum*, tandis que les mutations au niveau des codons S436A, A437G, K540E, A581G et A613S dans le gène DHPS, semblent jouer un rôle majeur dans le développement de la résistance au sulfadoxine chez *Plasmodium falciparum* (Berens et al. 2003, Jelinek et al. 1995, Jelinek et al. 1998, Sirawaraporn et al. 1997).

Il est rappelé que la SP est recommandée par l'OMS et toujours largement utilisée en Afrique pour le traitement préventif intermittent du paludisme chez les femmes enceintes (TPI) (Gosling et al. 2006, Schellenberg et al. 2005).

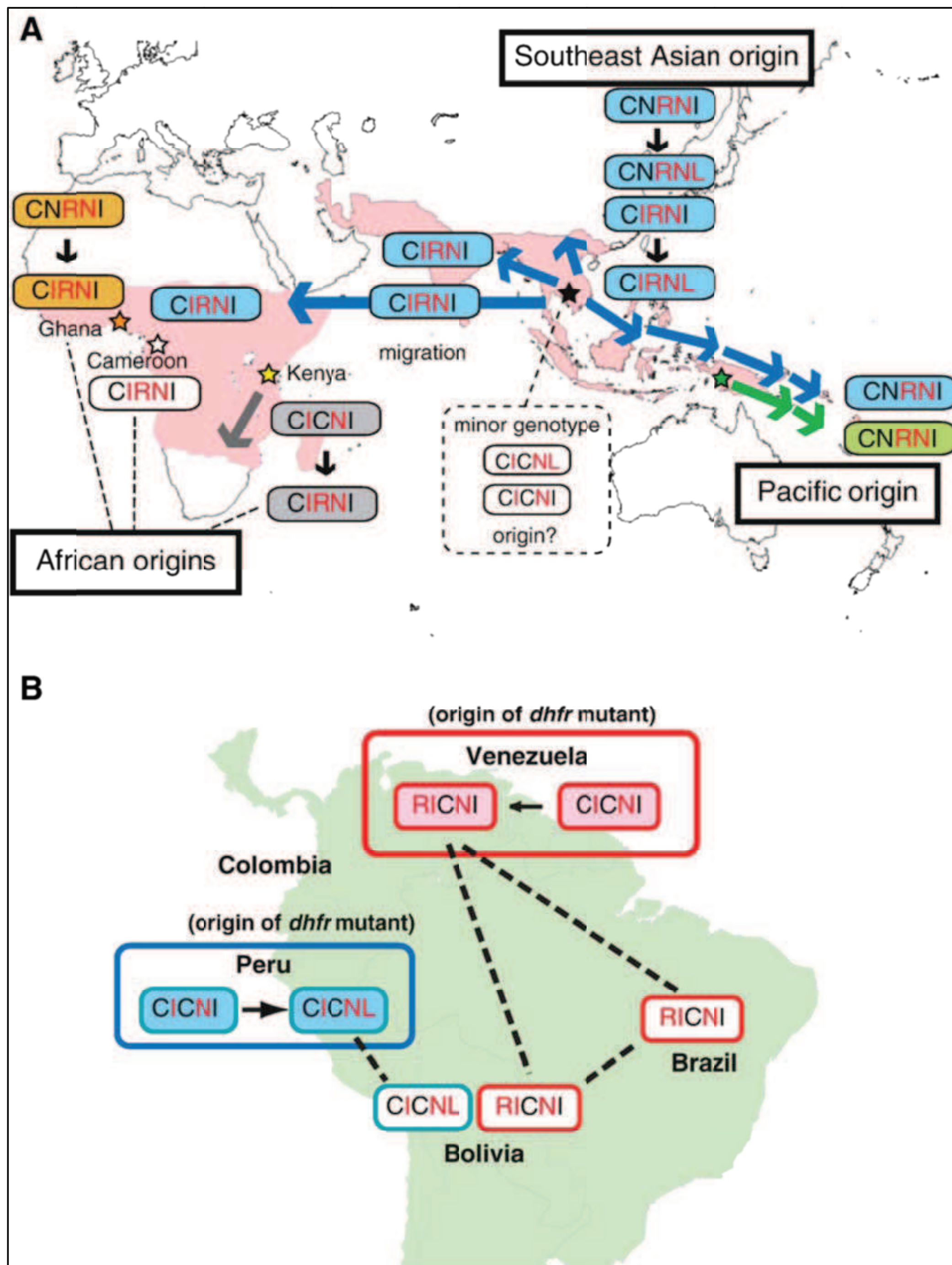

Figure 8 : Origine et diffusion de la résistance à la pyriméthamine (Mita et al. 2009).

A : Diffusion de la résistance à la pyrimethamine en Asie, en Afrique et dans la région Pacifique. La souche résistante CN**RN**I est probablement apparue dans une région proche de la frontière Khmero-Thaillandaise, aux cours des années 1960s. Elle a évolué en triple mutant C**IRN**I ou CN**RNL,** puis vers quatruble mutant C**IRNL.** La souche a diffusé vers les autres régions d'Asie et d'Afrique. Trois lignées autochtones du gène *Pfdhfr* ayant une triple mutation ont été caractérisées en Afrique. Dans la région Pacifique, deux lignées résistantes ayant l'haplotype CN**RN**I ont été observés: la lignée autochtone et la lignée qui a migré de l'Asie du Sud-Est.

B: Diffusion de la résistance à la pyrimethamine en Amérique du Sud. Deux lignées distintes de la résistance à la pyriméthamine ont eté détectées au Venezuela et au Pérou. Les deux lignées ayant triples mutants **RI**C**N**I et C**I**C**NL** sucessivement évoluées à partir des lignées de double mutant C**I**C**N**I du gène *Pfdhfr*.

2.4.4. Méfloquine (MQ)

La méfloquine, médicament schizonticide utilisé dans le traitement du paludisme *Plasmodium falciparum*, est un arylaminoalcool, introduit dans les années 1980s. Elle reste l'une des molécules recommandée pour la prophylaxie en zone de polyrésistance. La MQ a été utilisée comme le traitement de première ligne du paludisme simple en Thaïlande et au Cambodge après la QN (Pradines Bruno et al. 2010a) dans les années 1990.

La résistance à la MQ a été rapportée pour la première fois en 1991 (Looareesuwan et al. 1996, Nosten et al. 1991). Depuis, il a été observé une diminution de son efficacité dans certaines régions d'Asie du Sud Est, mais également en Papouasie-Nouvelle-Guinée, en Afrique, au Brésil (Fontanet et al. 1993). Depuis 2001, son association avec l'artésunate reste très largement utilisée, en Asie (AS-MQ). Plusieurs études (cliniques ou *in vitro*) ont montré une corrélation entre la résistance à la MQ et l'amplification du nombre de copies de *Pfmdr-1.* Cette amplification est aussi associée à un risque significativement élevé d'échecs thérapeutiques à l'association artesunate-méfloquine (Lim et al. 2009, Picot et al. 2009). Il est important à noter que les différentes copies du gène *Pfmdr-1* sont identiques et correspondent à l'allèle sauvage (Duraisingh et al. 2000).

2.4.5. Atovaquone (ATV)

L'atovaquone (ATV) est un hydroxy-1-4-naphthoquinone, analogue d'ubiquinone possédant des activités anti-pneumocystiques. *Pfcytb* (situé dans le génome mitochondrial) code pour le cytochrome b qui est la cible moléculaire de l'atovaquone. L'analyse de la séquence en acides aminés constituant le cytochrome b plasmodiale montre qu'il diffère de celui des autres organismes (Vaidya et al. 1993). L'utilisation de l'ATV dans les accès simples de *P. falciparum,* montre une bonne efficacité, mais ce traitement est souvent associée à un taux de recrudescence élevé pouvant atteindre les 30 % (Chiodini et al. 1995, Looareesuwan et al. 1996). Afin d'éviter l'apparition rapide de résistances à l'ATV, cette molécule a été associé au proguanil (un antifolate) et est commercialisée sous le nom de Malarone®. Le proguanil, qui seul n'a aucune action sur le potentiel membranaire de la mitochondrie, augmente la capacité de l'ATV à perturber ce potentiel (Srivastava and Vaidya 1999). Les mutations Y268N et Y268S dans le gène *Pfcytb* sont fortement associées à la résistance du parasite à cette molécule (Ekala et al. 2007, Musset et al. 2006).

2.4.6. Dérivés de l'artémisinine et ACTs

En 2001, l'Organisation Mondiale de la Santé a recommandé que les combinaisons thérapeutiques à base d'artémisinine (ACT) soient adoptées pour le traitement en première ligne du paludisme non compliqué

à *Plasmodium falciparum* (WHO 2010). Cette recommandation a été décidée suite à l'émergence et de la diffusion globale de la résistance aux antipaludiques classiques tels que la chloroquine et la sulfadoxine-pyriméthamine (Lin et al. 2010, Snow et al. 2001).

L'artémisinine (ART) est un alcaloïde naturel extrait de l'armoise *Artemisia annua* qui est utilisée en Chine depuis plus de 2 000 ans (Pradines Bruno et al. 2010a) pour le traitement des maladies cutanées ou du paludisme. Elle n'a été étudiée en Occident qu'à partir des années 1970 et introduite dans la pharmacopée dans les années 1990 (Maude et al. 2009, White 2008). Son action semble être liée à sa forte activité oxydante. Son temps de présence dans le sang est assez court (C_{max} par voie orale < 2 h et demi-vie < 1 h) (Pradines Bruno et al. 2010a). Cliniquement, l'artémisinine utilisée en monothérapie est uniquement curative lorsqu'elle est administré pendant 7 jours (Li et al. 1994, Stepniewska et al. 2010). Elle agit très vite sur la charge parasitaire mais elle ne permet pas d'éliminer complètement tous les parasites. Par conséquent, les combinaisons intégrant une drogue partenaire (ACT) ont été commercialisées (artésunate-sulfadoxine/pyriméthamine, artésunate-amodiaquine, artémether-luméfantrine, artésunate-méfloquine, artésunate-chloroproguanil/dapsone, artésunate-atovaquone/proguanil, dihydroartemisinin-pipéraquine et artésunate-pyronaridine) (Figure 9) (O'Brien et al. 2012). Les ACTs ont une activité inhibitrice sur toutes les étapes du développement parasitaire de stade intra-érythrocytaire (de l'anneau immature jusqu'au stade de trophozoïte plus mature), et également sur le développement au stade sexué des parasites immature (gamétocytes) (Price et al. 1996).

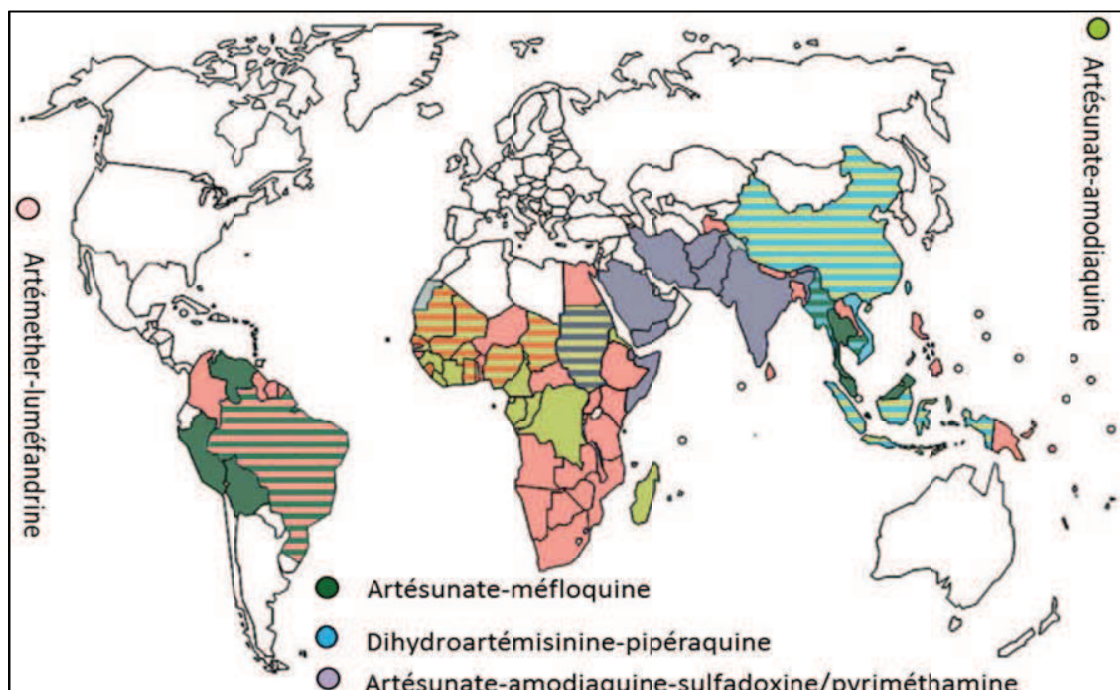

Figure 9 : Distribution des combinaisons thérapeutiques à base de l'artémisinine comme le traitement en première ligne à Plasmodium falciparum non compliqué dans le monde (O'Brien et al. 2012)

Cependant, les premiers cas d'échecs cliniques aux dérivés de l'artémisinine ont été identifiés en Asie du Sud-est, dans la même région où les premiers cas de résistance à la chloroquine, à la sulfadoxine-pyriméthamine et à la méfloquine ont été décrits (Dondorp et al. 2009, Noedl et al. 2008, Roper et al. 2004, Wellems and Plowe 2001, Wongsrichanalai and Meshnick 2008, Wongsrichanalai et al. 2002, Wootton et al. 2002). L'émergence de la résistance à l'artémisinine semble être actuellement limitée au bassin du Mékong (Ashley et al. 2014). Sa détection est clinique et est exprimé par un délai de clairance des parasites allongée au cours des 3 jours de traitement (Figure 10).

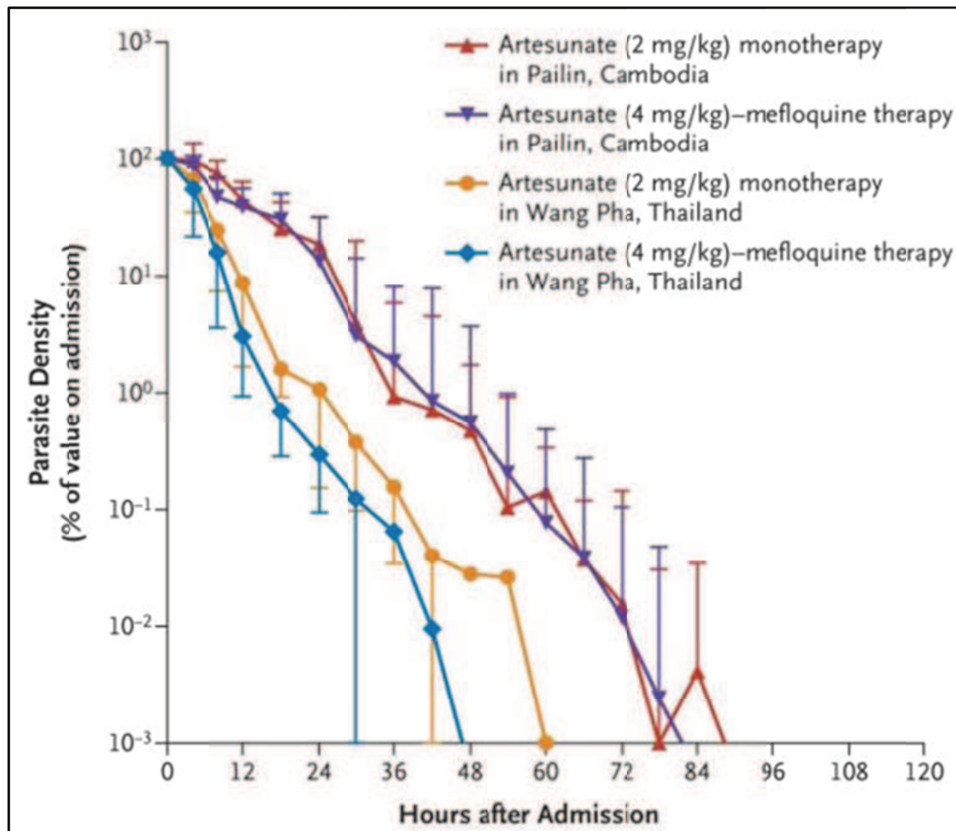

Figure 10. Delai de clairances parasitaires chez des patients Cambodgiens (région de Pailin) et des patients thaillandais (Mae Sot) au cours des 3 jours de traitement à l'artésunate (Dondorp et al. 2010). Les densités parasitaires par groupe de traitement sont les médianes normalisées en Log_{10}. Le barres verticales représentent les demi-interquartiles. La sensibilité de l'artésunate des souches de *Plasmodium falciparum* venant de Pailin est réduite comparée avec celle de Wang Pha en Thaillande. La résistance clinique n'était pas corrélée avec les données in vitro, utilsant le test classique à l'hyoxanthine titriée (Dondorp et al. 2010) .

Cette émergence est inquiétante sachant que nous ne disposons pas d'outils plus performants pour la détecter (phénotype *in vitro* et marqueur moléculaire) et l'arsenal thérapeutique contre le paludisme ne dispose d'aucune molécule pouvant remplacer les ACT.

3. Le paludisme au Cambodge

3.1. Epidémiologie du paludisme

Le Royaume du Cambodge, est un pays agricole de 181 035 kilomètres carrés, entouré par la Thaïlande à l'Ouest et au Nord, par le Laos au Nord et par le Vietnam à l'Est. Le pays possède 443 kilomètres de côtes, au Sud-Est, le long du golfe de Thaïlande. La région centrale du pays est marquée par la présence du lac Tonlé Sap. Les frontières au Nord et à l'Ouest du pays sont bordées de crêtes montagneuses. Malgré le développement important des infrastructures de communication ces dernières années, certaines zones de collines restent très isolées. Le climat de mousson est représenté par deux saisons, la saison humide et la saison sèche, qui ont la même durée de 6 mois. La forêt couvre environ les deux tiers du pays, même si elle est fortement dégradée par les incendies et par l'exploitation agricole du territoire.
La population du Cambodge est actuellement à 15,47 million d'habitants (http://worldpopulationreview.com/countries/cambodia-population/).

Le Cambodge avec la Birmanie est l'un des pays en Asie du Sud-Est où la transmission du paludisme est la plus importante. Les populations vivant dans les villages au bord des forêts ou ayant des activités liées à la forêt sont les plus à risque de contracter le paludisme. Le Ministère de la Santé a créé une institution spécialisée, le « Centre National de Malariologie (National Center for Malariology) » pour mettre en place, à l'échelle nationale, les stratégies de lutte contre le paludisme.

Environ 44% de la population cambodgienne vit dans les zones de forte transmission palustre (WHO 2013). Entre 2001 et 2009, le nombre de cas de paludisme détectés par le système de santé officiel est passé de 121 612 à 80 644 cas. Il a encore baissé en 2010 avec 44 659 cas (WHO 2009, 2010). Les cinq espèces de *Plasmodium* connus pour provoquer le paludisme chez l'homme ont déjà été décrites au Cambodge, y compris *P. knowlesi* en 2010 (Khim et al. 2011). En 2012, la proportion des cas de paludisme à *P. falciparum* était de 56%, alors que la proportion des cas de paludisme à *P. vivax* était de 44% (WHO 2013). Dans les régions rizicoles, au Sud et au Centre, la transmission est faible ou non existante. L'incidence de paludisme est plus élevée dans les provinces de l'Est à Mondulkiri et à Ratanakiri (Delacollette et al. 2009).

Le déclin du nombre des cas et de décès dû au paludisme (Figure 11) rapportés au cours des 11 dernières années est lié à plusieurs facteurs tels que la stabilité politique, le développement économique, la diffusion des informations, les changements dans les expositions professionnelles, les changements de l'environnement, et surtout l'amélioration de l'accès aux outils de lutte contre le paludisme organisé par le CNM et les autres institutions partenaires. Néanmoins, l'incidence de paludisme (les cas traités dans les centres de santés publiques) reste toujours élevée et continue d'être une cause majeure de santé publique et

un fardeau économique pour le Cambodge. Cette incidence était estimée à 4.07 cas pour 1000 habitants en 2010.

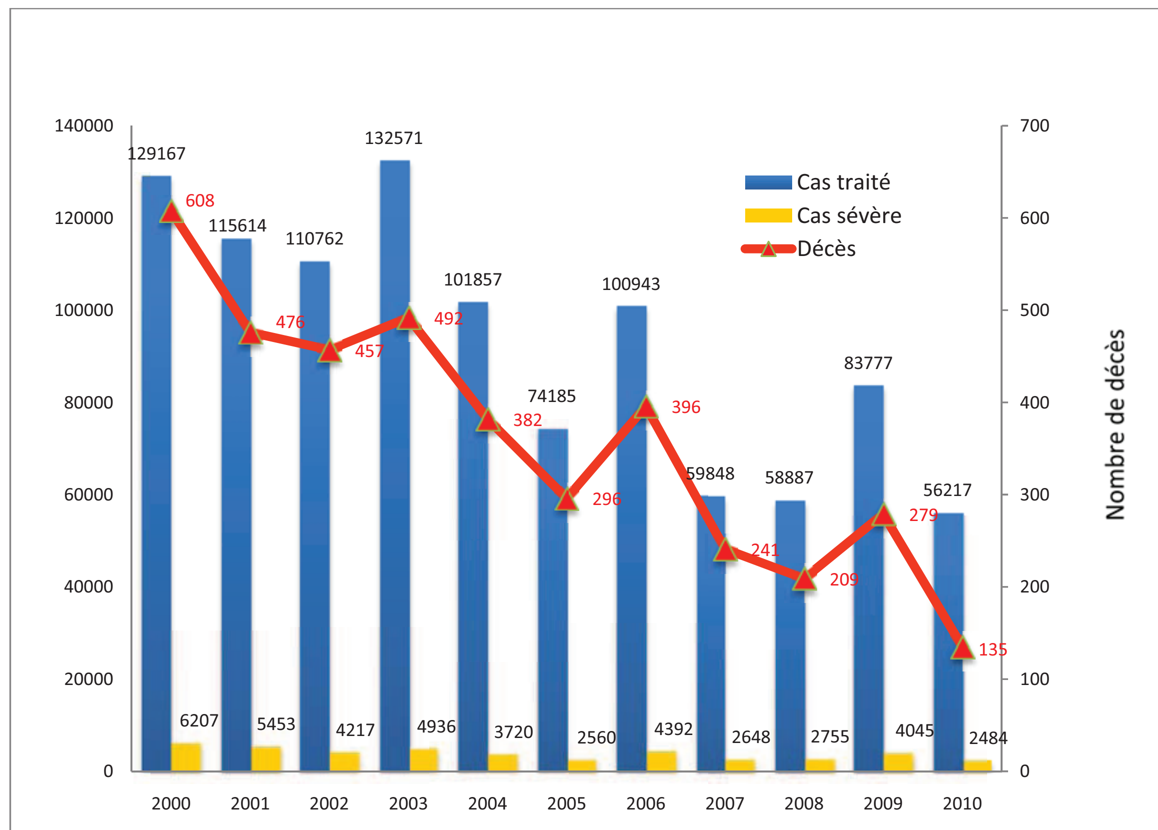

Figure 11: Estimation de la morbidité et de la mortalité liés au paludisme au Cambodge entre 2000 et 2010 (Source Rapport annuel CNM, 2012).

3.2. Les vecteurs du paludisme au Cambodge

Entre 1959 et 1963, les principaux vecteurs du paludisme au Cambodge étaient *Anopheles dirus* s.l., *Anopheles minimus* s.l., *Anopheles maculatus* and *Anopheles sundaicus* s.l (Garros et al. 2006, Socheath et al. 2000). Aucune étude de cette envergure n'a ensuite pu être menée durant 25 ans à cause des problèmes socio-politiques du pays. En 1997, de nombreuses espèces de moustiques anophèles ont été rapportées à Kampong Speu et à Kratie (19 et 25 espèces respectivement) parmi lesquels celles déjà décrites comme *An. dirus* s.l, *An. minimus* s.l and *An. Maculatus* (Socheath et al. 2000).

3.2.1. Distribution des vecteurs et le comportement des espèces Anophèles au Cambodge

La couverture forestière est un facteur très important dans l'évaluation du risque du paludisme, même si ce paludisme couvre un spectre épidémiologique large en raison de la diversité des espèces, de la démographie humaine et des comportements humains (Schapira and Boutsika 2012). La transmission du

paludisme au Cambodge est étroitement associée à deux vecteurs qui habitent dans la forêt et au bord de la forêt.

An. dirus réside dans la zone avec prédominance de la forêt. *An. minimus*, qui est un vecteur du paludisme moins efficace, s'installe lui dans et autour des rizières en bordure de forêt (Garros et al. 2006, Trung et al. 2004). *An. dirus*, *An. minimus* et *An. maculatus* piquent principalement à l'extérieur des maisons (Trung et al. 2005). Cette tendance exophagique est associée à la persistance de la transmission du paludisme parmi les populations ayant des activités de plein air pendant la nuit. *An. dirus* au Cambodge est le plus anthropophile comparé aux autres espèces vectorielles (Trung et al. 2005). Le taux d'inoculation d'*An. dirus* a été estimé à plus de 1% (taux de CSP) à Ratanakiri ce qui indique que cette espèce est un vecteur très efficace et joue un rôle important pour la transmission du paludisme (Trung et al. 2004).

An. minimus préfère se nourrir sur les animaux plutôt que sur l'homme (Trung et al. 2005). La répartition et l'abondance ces vecteurs a cependant changé en raison de l'exploitation intensive des terres cultivables, de la déforestation, du changement climatique et peut-être à cause des insecticides utilisés dans le cadre de la lutte anti-vectorielle dans les zones endémiques paludéennes (Garros et al. 2006, Guerra et al. 2006, Obsomer et al. 2007, Van Bortel et al. 2008). Le complexe *dirus* au Cambodge est représenté seulement par *An. dirus*.

An. sundaicus s.l. et *An. epiroticus* (*An. sundaicus* A) ont été retrouvé dans les régions côtières du Sud au Cambodge. D'autres espèces ont également été décrites comme *An. annularis* s.l., *An. barbirostris* s.l, *An. culicifacies* B (Van Bortel et al. 2002). Au sein du groupe *maculatus*, une étude récente a montré pour la première fois la présence d'*An. Sawadwongporni* à Kampong Speu (Suwonkerd et al. 2013).

L'abondance des vecteurs du paludisme au Cambodge est spécifiquement lié aux sites. Par exemple à Pailin, parmi les trois espèces principales, *An. minimus* (67.2%) a été trouvé plus fréquemment qu'*An. maculatus* (20.6%) ou *An. dirus* (9.9%), tandis qu'à Pursat, la fréquence d'*An. dirus* était de 52%, probablement influencé par les conditions environnementales locales et la topographie des lieux (Sochantha et al. 2010). Une étude récente menée à Pailin et à Pursat a montré une réduction de près 45% de la fréquence des repas sanguins d'*An. minimus* dans deux villages après l'introduction de hamacs imprégnés d'insecticide à long durée d'action (LLIH) (Sochantha et al. 2010). Cependant, le risque lié à l'utilisation régulière d'insecticide est le développement de la résistance aux insecticides dans les populations vectorielles. Néanmoins, jusqu'à présent, la résistance aux insecticides n'est pas un problème majeur pour les vecteurs primaires du paludisme, comme *An. dirus* et *An. minimus*. Ces deux espèces restent sensible à la perméthrine, sauf dans une seule région d'étude au Cambodge où *An. dirus* était résistant au DDT (Van Bortel et al. 2008).

3.2.2. *Implication de l'évolution des conditions sociales et environnementales sur les vecteurs et la transmission*

Les facteurs environnementaux ont un certain impact sur la distribution et le comportement des espèces vectrices du paludisme (Manguin et al. 2008). *An. dirus* vit en zone forestière, mais dispose d'une capacité d'adaptation aux changements environnementaux allant des habitats forestiers naturels à la forêt cultivée, comme les plantations de caoutchouc, de thé et d'autres types de vergers (Gingrich et al. 1990, Rosenberg et al. 1990, Suwonkerd et al. 2002). La déforestation, causée par des activités humaines très diverses, comme l'exploitation forestière, le défrichement pour le développement agricole, les programmes de transmigration, les constructions routières, l'exploitation minière et le développement hydroélectrique (Patz et al. 2000, Walsh et al. 1993), est un des facteurs majeurs associés à l'émergence et à la propagation des maladies infectieuses, comme le paludisme (Vanwambeke et al. 2007, Yasuoka and Levins 2007). La plupart des espèces vectrices du paludisme sont sensibles à ces changements environnementaux (Suwonkerd et al. 2002). Le défrichement extensif de la forêt a eu un impact énorme sur les écosystèmes, en particulier la modification de microclimats en réduisant l'ombre, l'humidité et la pluviométrique (Overgaard et al. 2002, Reiter 2001).

Les stratégies de lutte anti-vectorielle à grande échelle qui ont eu lieu au cours des dernières années au Cambodge, étaient basées sur la distribution gratuite de moustiquaires imprégnées d'insecticide à longue durée d'action. Cela a contribué à une diminution substantielle des cas de paludisme. Toutefois, l'impact des moustiquaires reste limité sur les principaux vecteurs comme *An. dirus s.s.* et *An. minimus s.s.*, espèces exophagiques et exophiles. La transmission du paludisme intervenant en début de la soirée a bien décrite à l'Ouest du Cambodge (Sochantha et al. 2010), prouvant que des mesures de contrôle anti-vectorielle supplémentaires sont nécessaires.

3.3. Epidémiologie de la résistance aux antipaludiques

La frontière Khmero-Thaïlandaise est une zone clé dans l'émergence de la résistance aux antipaludiques. Cette région a été la première où des parasites résistants (*Plasmodium falciparum)* à la chloroquine, à la sulfadoxine-pyriméthamine et à la méfloquine ont été décrits. La résistance aux deux premiers médicaments s'est ensuite diffusée dans la région Mékong supérieur puis à travers l'Inde en Afrique.

Le CNM, en charge de la lutte contre paludisme, évalue et surveille régulièrement depuis 1991, l'efficacité des antipaludiques dans le traitement du paludisme à *Plasmodium falciparum*. La méfloquine utilisée en monothérapie était le traitement recommandé entre 1993 et 2000. Après la découverte de parasites résistants à cette drogue, en 2000, la stratégie de traitement utilisé en première ligne a été changée avec l'introduction de la combinaison artésunate-méfloquine en traitement de 3 jours (WHO 2010).

A cette époque, le Cambodge figurait parmi les premiers pays à adopter les combinaisons thérapeutiques à base d'artémisinine comme le traitement en première ligne dans la stratégie nationale (WHO 2010). Depuis cette date, le CNM en collaboration avec l'OMS a conduit plusieurs études visant à évaluer l'efficacité thérapeutique de l'artésunate-méfloquine (suivi des malades traités jusqu'à 28 jours ou 42 jours). Les études menées entre 2001 et 2004, ont montré que la fréquence des échecs thérapeutiques était inférieure à 5% dans la plupart de sites. Toutefois, certaines données de Pailin en 2002, ont montré que 14,3% des patients traités étaient en échec thérapeutique au bout de 28 jours. En 2004, un pourcentage élevé d'échec thérapeutique (9,9%) a été confirmé (Denis et al. 2006b). Compte tenu que la résistance à la méfloquine avait déjà émergé dans cette zone, il a été initialement considéré que les échecs thérapeutiques étaient dû à la diminution d'efficacité de la méfloquine. Néanmoins, il a été constaté par la suite que près de 10% des patients n'avaient pas été éliminé les parasites au jour 3 (Alker et al. 2007) et que les temps de clairance parasitaire étaient beaucoup plus long que ceux rapportés antérieurement (Price et al. 1997). A peu près au même temps, entre 2001 et 2003, l'efficacité de la combinaison artéméther-luméfandrine a été étudiée à Battambang, dans le Nord-Ouest du Cambodge (Denis et al. 2006a). Les données obtenues montraient que 13,8% à 32,7% des patients présentaient les parasites au jour 3, alors que pendant la même période, à Pailin, la fréquence des taux d'échec thérapeutique chez les sujets traités par la combinaison artésunate-méfloquine diminuaient de 9.9 à 14.3% en 2002-2004 à 0-0.5% en 2007-2008, possiblement lié à la mise en place des tests de diagnostic rapide et au remplacement de la combinaison artésunate-méfloquine par la combinaison dihydroartémisinine-pipéraquine au niveau communautaire (WHO 2010). Ces résultats cliniques étaient confirmés par la biologie moléculaire montrant une proportion importante de parasites ayant un nombre de copies élevés du gène *Pfmdr-1* (Hunt et al. 2010).

La résistance à l'artémisinine n'a été rapportée qu'en 2008. Il a été fourni par une étude menée par l'Institut des Forces Armés pour la recherche des Sciences médicales utilisant l'artésunate en monothérapie (Noedl et al. 2008). Les patients atteints de paludisme dans la région de Tasanh, province de Battambang (au Sud de Pailin), étaient admis à l'hôpital pendant 28 jours. Ils étaient traités soit avec l'artésunate seule (4 mg/kg de poids corporel par jour pendant 7 jours) soit avec de la quinine (30 mg/kg de poids corporel par jour) plus tétracycline (25 mg/kg de poids corporel par jour) en une dose fractionné toutes les 8 h pendant 7 jours. Quatre échecs thérapeutiques ont été observés parmi les 60 patients ayant reçu l'artésunate. Parmi les quatre patients, deux avaient une concentration sanguine adéquate en artésunate. Ces 2 patients ont été classés comme présentant une infection résistante à l'artésunate. Leur temps de clairance parasitaire étaient respectivement de 133 h et de 95 h, largement plus longue que la médiane des temps de clairance parasitaire observés (52,2 h). La concentration inhibitrice 50% (CI_{50}) à la dihydroartémisinine des parasites isolés chez ces 2 patients était quatre fois supérieure à la moyenne géométrique de CI_{50} des patients guéris et plus de dix fois supérieure à celle obtenus avec le clone référence W2. Au cours du suivi des patients inclus dans l'étude, il a été observé que 47,9% et 21,9% d'entre eux, ayant reçu de l'artésunate monothérapie par voie orale, avaient encore des parasites présents dans la circulation sanguine, 48h et 72 h après le début du traitement. Bien que seulement deux échecs thérapeutiques (3.3%) répondaient aux critères de la définition de la

résistance à l'artémisinine, les résultats observés semblaient confirmer les études menées entre 2002 et 2004 par le CNM.

Depuis 2009, il a été constaté, à Pailin, que la proportion des patients encore parasitémiques au jour 3 après traitement avec la combinaison dihydroartémisinine-pipéraquine avait augmenté de 26% à 33% entre 2008 et 2009 (WHO 2010). A Pailin et à Tasanh, plus de 40% des patients présentaient une parasitémie au jour 3 après le traitement avec l'artésunate monothérapie par voie orale (2-4 mg/kg du poids corporel par jour). Quelques échecs thérapeutiques tardifs ont été observés dans ces deux sites, alors que les patients présentaient une concentration plasmatique adéquate d'artésunate ou de dihydroartémisinine. Cependant aucune équipe n'a réussi à montrer clairement une réduction de la sensibilité des parasites en utilisant les tests *in vitro* disponibles. Par ailleurs, à la frontière entre la Thaïlande et la Birmanie, 10-20% des patients ont été trouvés positifs au jour 3 après avoir traité avec un ACT. Enfin, les dernières études menées à la frontière entre la Chine et la Birmanie et utilisant de l'artésunate par voie orale en monothérapie (16 mg/kg du corporel par jour pendant 7 jours) ont montré que 25% des patients étaient parasitémiques au jour 3 (WHO 2010).

OBJECTIFS ET RESULTATS

Les principaux objectifs de ce travail ont été :

OBJECTIF 1 : d'évaluer l'impact des stratégies mises en place pour lutter contre le paludisme à P. falciparum sur les autres espèces de Plasmodium présentent au Cambodge (***articles 1, 2 et manuscrit en préparation 1***);

OBJECTIF 2 : de mettre en place des outils biologique et moléculaires, permettant de mieux définir l'épidémiologie des parasites résistants, en particulier la quinine (***article 3 et 4***) et aux dérivés de l'artémisinine (***articles 5, 6 et 7***) ;

OBJECTIF 3 : d'étudier et de définir la structuration des populations parasitaires circulant au Cambodge pour estimer les zones à risque de diffusion de la résistance à l'artémisinine, en utilisant des approches génomique et bio-informatique (***manuscrit en préparation 2***).

OBJECTIF 1: Evaluation de l'impact des stratégies mises en place pour lutter contre le paludisme à *P. falciparum* sur les autres espèces de *Plasmodium*

O1.1. Etude de la diversité génétique de polymorphismes du gène *dihydrofolate reductase* chez *Plasmodium malariae* et *Plasmodium ovale*

Les paludismes à *Plasmodium malariae* ou *Plasmodium ovale* ont longtemps été considérés comme une maladie à faible prévalence avec une distribution géographique limitée et bénigne. Par conséquence, peu d'attention a été porté à ces deux espèces minoritaires souvent associés à *Plasmodium falciparum* ou/et à *Plasmodium vivax*. De ce fait, ces parasites sont souvent soumis à l'action des traitements antipaludiques pour traiter le paludisme à *P. falciparum* ou *P. vivax* dans les régions de co-endémicité. A titre d'exemple, au Cambodge, à part de donnée publiée par (Incardona et al. 2005), qui a décrit la présence de *Plasmodium ovale* au sein des populations vivant dans les villages du Nord-Est du Cambodge (détection par PCR spécifique d'espèce ciblant le gène SSUrRNA), peu de données sont disponibles.

Le développement de la résistance est souvent considéré comme se déroulant en deux phases. Dans la première phase, un évènement génétique initial se produit et un mutant résistant émerge et est sélectionné positivement, le nouveau trait génétique donnant un avantage pour sa survie en présence de drogues antipaludiques. La deuxième phase, comprend la multiplication des parasites résistants sélectionnés par la pression médicamenteuse et l'émergence d'une population parasitaire insensible au traitement. Les analyses moléculaires, basée sur l'étude d'isolats de terrain ou de souches de laboratoire, ont démontré que la résistance à la pyriméthamine chez *Plasmodium falciparum* était associée à des mutations ponctuelles spécifiques au sein du gène *dihydrofolate reductase* (*Pfdhfr*) (Peterson et al. 1990, Plowe et al. 1998). Il a été démontré que la présence de la mutation S108N chez les patients infectés par *Plasmodium falciparum* augmentait le risque l'échec thérapeutique de la pyriméthamine (Gregson and Plowe 2005).

Dans l'***article 1***, présenté ci-dessous, notre objectif était d'améliorer notre connaissance concernant le polymorphisme du gène *dhfr* chez *P. malariae*, en poursuivant les travaux initiés par Tanomsing et collaborateurs qui avait publié 35 séquences du gène *Pmdhfr* provenant majoritairement de l'Afrique (Tanomsing et al. 2007). Nous avons donc séquencé le gène *Pmdhfr* de 123 nouveaux isolats venant d'Afrique, du Cambodge et de Madagascar. Nous avons choisi d'inclure des isolats de Madagascar en raison de l'utilisation l'association Sulfadoxine-Pyriméthamine (SP) dans le traitement preventif intermitent chez les femmes enceintes. Nous avons mis en place une méthodologie d'étude applicable aux échantillons pour lesquels nous disposons d'information sur leur origine (géographie, date d'isolement). Les échantillons cambodgiens ont été isolés à partir de la bio-banque de l'Institut Pasteur du Cambodge qui a été constitué à

partir de 2001 dans l'Unité Epidémiologie Moléculaire du Paludisme. Cette bio-banque a été constituée autour de plusieurs projets. En parallele nous avons utilisé les ADN fournis par d'autres instituts ou centres de recherche. Au final, nous avons trouvé que 2 mutations (sur un total de 8) au sein du gène *Pmdhfr* pouvaient être lié à la résistance à la pyriméthamine chez *Plasmodium malariae* : la mutation S58R correspondant à la mutation C59R chez *P. falciparum* ou à la mutation S58R chez *P. vivax* (isolats malgaches) et la mutation S114N correspondant à la mutation S108N chez *P. falciparum* ou à la mutation S117N chez *P. vivax* (3 échantillons cambodgiens). Les échantillons provenant de pays Africains étaient tous de type sauvage. Trois autres mutations proches de SNP associée à la résistance à la pyriméthamine (K55E, S59A et F168S) ont aussi été détectés parmi les échantillons malgaches. Il est important de noter qu'à Madagascar, nous avons retrouvés un polymorphisme plus elévé au sein du gène *Pmdhfr*, probablement lié à la pression de la pyriméthamine exercée avec l'augmentation d'utilisation de SP depuis la mise en oeuvre des traitements préventifs intermittants pour les femmes enceintes (TPI) (Menard et al. 2008).

Article 1: **Khim, N.**, S. Kim, C. Bouchier, M. Tichit, F. Ariey, T. Fandeur, P. Chim, S. Ke, S. Sum, S. Man, A. Ratsimbasoa, R. Durand and D. Menard (2012). Reduced impact of pyrimethamine drug pressure on Plasmodium malariae dihydrofolate reductase gene. Antimicrob Agents Chemother 56: 863-868.

Dans le ***manuscrit en préparation 1***, l'étude de la diversité génétique du gène *dhfr* chez *Plasmodium ovale* à partir de 83 isolats (dont 11 prélèvements du Cambodge) nous a permis de montrer que le gène *dhfr* pouvait être utilisé a) pour étudier la prévalence des mutations au sein du gène *dhfr* de *Plasmodium ovale* liées à la résistance à la pyriméthamine (comparaison avec des gènes orthologues de *P. falciparum, P. vivax* et *P. malariae*) et b) pour caractériser quelle sous espèce de *Plasmodium ovale* est présente dans les populations parasitaires cambodgiennes. Dans cette étude, 4 isolats provenant du Cameroun ont été classés comme mutant (S58**R**) (*Plasmodium ovale curtisi),* mutation correspondant à la mutation C59**R** chez *Plasmodium falciparum* ou à la mutation S58**R** chez *Plasmodium vivax* et S58**R** dans *Plasmodium malariae*. Les 11 isolats cambodgiens ont été identifiés comme *Plasmodium ovale curtisi* (type classique). Nous avons pu en revanche observer la distribution des 2 sous espèces de *P. ovale (Plasmodium ovale curtisi et Plasmodium ovale wallikeri)* au sein de différentes populations circulant au Cameroun, au Mali, en République Centrafricaine, aux Comores, au Congo, en Côte d'Ivoire, ou à Sao Tome et Principe.

Commentaires

Dans les deux articles présentés ici, j'ai travaillé sur 6000 échantillons cambodgiens collectés entre 2001 et 2012, conservés à -20°C. La présence de *Plasmodium spp*. a été réalisée en utilisant plusieurs types de Nested-PCR ou de PCR en temps réel dirigées contre les gènes *18S* ou *cyt b* (Singh et al. 1999, Steenkeste et al. 2009). Nous avons obtenu environ 2000/6000 échantillons positifs. Ces résultats ont été publiés dans d'autres études (Canier et al. 2013, Hoyer et al. 2012, Incardona et al. 2005, Khim et al. 2005, Khim et al. 2011, Mueller et al. 2014, Steenkeste et al. 2009).

Dans le cadre de ma thèse, nous avons pu détecter 26 et 11 isolats infectés par *Plasmodium malariae* et *Plasmodium ovale* respectivement, dont deux échantillons présentant une infection mixte (en association avec *P. falciparum* et *P. vivax*). Le gène de la *dihydrofolate reductase* a été amplifié dans 204 échantillons nous permettant d'obtenir 123 séquences de *Pmdhfr* et 83 séquences de *Podhfr*. Les produits PCR *Pmdhfr* et *Podhfr* ont été ensuite envoyés au séquençage à la Génopôle, Paris, France. Enfin, les séquences d'acide aminé de *Pmdhfr* ont été alignées avec les séquences sauvages du même gène chez *Plasmodium falciparum* et *Plasmodium vivax*. Tandis que toutes les séquences disponibles de *Podhfr* ont été comparées avec les séquences sauvages chez *Pfdhfr, Pvdhfr, Pmdhfr*.

Nous avons pu récupérer des ADN d'isolats de *Plasmodium malariae* collectés au Cambodge, à Madagascar, et d'autre pays en Afrique. L'Institut Pasteur de Madagascar nous a fourni 53 échantillons contenant de l'ADN de *Plasmodium malariae* et le Centre National de Référence du Paludisme en France nous a fourni 44 *Plasmodium malariae* et 72 *Plasmodium ovale*.
Notre stratégie d'étude a reposé sur la comparaison des séquences du gène *dhfr* dans des isolats d'origine géographique différente pour prendre en compte les variations locales concernant la pression de sélection appliquée aux populations de *Plasmodium.*

Amplification, séquençage, analyse de séquence

123 isolats de *Plasmodium malariae* comprenant 26 isolats cambodgiens, 53 isolats venant de Madagascar, 44 isolats Africains et 83 isolats de *Plasmodium ovale* dont 11 du Cambodge et 72 des pays d'Afrique ont été amplifiés et séquencés pour le gène *dihydrofolate reductase* en utilisant la même stratégie validée dans (Khim et al. 2012). Leurs séquences ont été obtenues par la méthode décrite par Sanger. Les analyses des polymorphismes de séquences *Pmdhfr* et *Podhfr* ont été faites en utilisant le logiciel CEQ2000 et comparées avec les séquences publiques de *Pmdhfr* (AY846633), de *Plasmodium ovale wallikeri* (EU266601) et de *Plasmodium ovale curtesi* (EU266606). Les séquences d'acide aminé ont été alignées avec les séquences sauvages (GenBank accession no. XM_001351443 pour *Pfdhfr*, X98123 pour *Pvdhfr* et AY846633 pour *Pmdhfr*) par le logiciel BioEdit Sequence Alignment Editor (Kawamoto et al. 1999). Les polymorphismes du gène *dihydrofolate reductase* chez *Plasmodium malariae* (*Pmdhfr*) et *Plasmodium ovale* (*Podhfr*) identifié à partir des isolats dans cette étude ont été alignés sur les séquences *Pmdhfr* et *Podhfr* respectivement disponible dans le GenBank.

Conclusion

Les polymorphismes du gène *Pmdhfr* semblent être faibles par rapport à ceux des espèces sympatriques de *Plasmodium*. Pourtant, selon nos données, parmi les isolats cambodgiens et malgaches, l'épidémiologie locale et l'histoire de la pression de la SP influence la sélection de mutation chez *dhfr*, comme les cas chez *Plasmodium falciparum* et *Plasmodium vivax*. De plus, l'amplification du gène *dihydrofolate reductase* chez *Plasmodium ovale* semble être utilisée pour identifier les sous espèces de *Plasmodium ovale*. 11 isolats

cambodgiens ont été identifiés comme *Plasmodium ovale curtisi* (type classique). Il s'agit de la première description des variantes de *Plasmodium ovale* au sein des populations parasitaires cambodgiennes, différentes des fréquences retrouvées dans les pays voisins. Des études supplémentaires en comparant les séquences du gène *dhfr* chez *Plasmodium spp*. à partir de la même population ou à partir de patient ayant des infections mixtes semblent indispensable.

Article 1

Antimicrobial Agents and Chemotherapy

Reduced Impact of Pyrimethamine Drug Pressure on Plasmodium malariae Dihydrofolate Reductase Gene

Nimol Khim, Saorin Kim, Christiane Bouchier, Magali Tichit, Frédéric Ariey, Thierry Fandeur, Pheaktra Chim, Sopheakvatey Ke, Sarorn Sum, Somnang Man, Arsène Ratsimbasoa, Rémy Durand and Didier Ménard
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Reduced Impact of Pyrimethamine Drug Pressure on *Plasmodium malariae* Dihydrofolate Reductase Gene

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Molecular investigations performed following the emergence of sulfadoxine-pyrimethamine (SP) resistance in *Plasmodium falciparum* have allowed the identification of the dihydrofolate reductase (DHFR) enzyme as the target of pyrimethamine. Although clinical cases of *Plasmodium malariae* are not usually treated with antifolate therapy, incorrect diagnosis and the high frequency of undetected mixed infections has probably exposed non-*P. falciparum* parasites to antifolate therapy in many areas. In this context, we aimed to assess the worldwide genetic diversity of the *P. malariae dhfr* gene in 123 samples collected in Africa and Asia, areas with different histories of SP use. Among the 10 polymorphic sites found, we have observed 7 new mutations (K55E, S58R, S59A, F168S, N194S, D207G, and T221A), which led us to describe 6 new DHFR proteins. All isolates from African countries were classified as wild type, while new mutations and haplotypes were recognized as exclusive to Madagascar (except for the double mutations at nucleotides 341 and 342 [S114N] found in one Cambodian isolate). Among these nonsynonymous mutations, two were likely related to pyrimethamine resistance: S58R (corresponding to C59R in *P. falciparum* and S58R in *Plasmodium vivax*; observed in one Malagasy sample) and S114N (corresponding to S108N in *P. falciparum* and S117N in *P. vivax*; observed in three Cambodian samples).

Currently, in many parts of the globe, the emergence and spread of malaria parasites resistant to various antimalarial drugs recommended by the international organizations remain major factors threatening control efforts (41). Among the five *Plasmodium* species that affect humans (40), resistant *Plasmodium falciparum* parasites were first selected by chloroquine (CQ), a highly effective, fast-acting, and inexpensive 4-aminoquinoline widely used for several decades, in Southeast Asia or South America (29, 44) in the 1960s. CQ-resistant parasites of the other *Plasmodium* species emerged much later: in 1989 for *Plasmodium vivax* (Papua New Guinea) (32) and in 2002 for *Plasmodium malariae* (Indonesia) (26). Following the introduction of the sulfadoxine-pyrimethamine combination (SP) to replace CQ as the first-line treatment for uncomplicated *P. falciparum* malaria, the same scenario was observed. SP-resistant *P. falciparum* parasites were first detected in the 1980s (9, 19). Molecular investigations, based on laboratory and field isolates, demonstrated later that the resistance of *P. falciparum* to pyrimethamine was mediated by specific point mutations in the dihydrofolate reductase gene (*dhfr*) (30, 31). Currently, it is assumed that pyrimethamine resistance is conferred by the stepwise selection of a series of nonsynonymous point mutations (codons 50, 51, 59, and 164) from the S108N single mutant allele (24). Parasites with the triple mutant allele (N51I C59R S108N) have markedly reduced *in vitro* susceptibility to pyrimethamine, and the presence of this allele in a *P. falciparum*-infected patient increases the risk of SP therapeutic failure (15). The additional mutation I164L confers on the quadruple mutant a high level of resistance to pyrimethamine (22) and abrogates the clinical efficacy of SP, as observed in Southeast Asia, South America (31), and Africa (25). Although *P. vivax* and *P. malariae* infections are not usually treated directly with SP, the high frequency of mixed infections, such as *P. falciparum*-*P. vivax* or *P. falciparum*-*P. malariae* infections, that are not detected by microscopy examination (27) has inevitably exposed a large number of non-*P. falciparum* parasites to antifolate therapy in many areas.

Comparison of the dihydrofolate reductase (DHFR) enzymes in *P. falciparum* and *P. vivax* showed that the DHFR domains were 77.3% identical and the active-site regions were strongly conserved (23). Moreover, sequencing of the *dhfr* gene in numerous *P. vivax* isolates collected in areas where SP was used to treat *P. falciparum* and alignment of these alleles with *P. falciparum dhfr* alleles have demonstrated that mutations in codons 57, 58, 61, 117, and 173 were involved in resistance to pyrimethamine and corresponded to codons 51, 59, 108, and 164 in *P. falciparum* (for a review, see reference 17). Heterologous expression studies later confirmed the role of these mutations in the resistance of *P. vivax* to pyrimethamine (17).

More recently, Tanomsing et al. conducted a study that aimed to determine whether SP pressure had selected pyrimethamine-resistant *P. malariae* parasites (37). By cloning, sequencing, and alignment of 35 *Plasmodium malariae* dihydrofolate reductase (*Pmdhfr*) sequences with the *Plasmodium brasilianum* dihydrofolate reductase (*Pbdhfr*) sequence, they observed several nonsynonymous mutations in five different codons (H22Q, K48E, N50K, S114N/G, and I170M). Three of these (codons 50, 114, and 170)

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corresponded to analogous positions known to be associated with pyrimethamine resistance in *P. falciparum* (residues 51, 108, and 164) and *P. vivax* (residues 117 and 173) (37). After this finding, Choowongkomon et al. demonstrated by computational analysis of interactions between DHFR inhibitors and the modeled structure of malaria parasite DHFR enzymes that certain residues in *P. malariae* DHFR (*Pm*DHFR) were associated with significant reductions in binding energy for pyrimethamine (I13, L45, N53, S117, and I170) (8). However, the data acquired on PmDHFR polymorphism remain patchy; they are based on a few isolates, collected essentially in Southeast Asian countries (30/35 isolates), where SP has not been used for several decades. In this context, this study presents additional comprehensive data on the genetic diversity of *Pm*DHFR by sequencing a large set of isolates (n = 123) collected in areas with different histories of SP use (mainland Africa, Madagascar, and Cambodia).

MATERIALS AND METHODS

Sample collection. *P. malariae* samples were collected in Cambodia, Madagascar, and other countries in Africa. Isolates from Cambodia (venipuncture blood and dried blood spots) were obtained from symptomatic or asymptomatic individuals at different sites in 2001, 2004, and 2007, following microscopy examination. Samples from Madagascar were collected, as part of the surveillance of antimalarial drug resistance, between 2006 and 2008 from symptomatic patients before treatment in eight different health centers, located in areas exhibiting the four epidemiological patterns of malaria transmission (2). Other samples, obtained in 2001 to 2009 from malaria-infected travelers of African origin returning to France from various African countries (Comoros, Côte d'Ivoire, Cameroon, Mali, Gabon, and Togo), were provided by the National Reference Center for Malaria (NRCM), Paris, France.

DNA extraction, PCR amplification, and sequencing. Parasite DNA was extracted directly from blood samples and dried blood spots by using a DNA blood kit (Qiagen, Germany) or the InstaGene matrix (Bio-Rad, Marnes la Coquette, France) according to the manufacturer's instructions. The genomic DNA samples were stored at −20°C until use. Parasite species were confirmed by nested PCR (35) (Cambodian samples) or by real-time PCR (10) (Malagasy samples and samples from the NRCM).

Nested-PCR approaches were used to increase the sensitivity of amplification. First rounds of PCR were performed in 25 μl (final volume) of a reaction buffer containing 2.5 μl DNA, 0.25 μM each primer (PmDHFR_PCR_F [5′-ATTCGACATATATGCCATCTG-3′] and PmDHFR_PCR_R [5′-CCTTCTTGCGTTTACTGAAA-3′]), 250 μM each deoxynucleoside triphosphate (dNTP) (Solis BioDyne), 2.5 mM $MgCl_2$, and 1.25 U *Taq* polymerase (FirePol DNA polymerase I; Solis BioDyne). Amplifications were performed under the following conditions: 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 52°C for 90 s, and 72°C for 120 s. DNA synthesis was achieved by final extension at 72°C for 10 min (770 bp). Nested PCRs were carried out in 55 μl (final volume) of a reaction buffer containing 2 μl of PCR products, 0.4 μM each primer (PmDHFR_Nest_F [5′-GCTTGCTGTAAAGTGCCAAA-3′] and PmDHFR_Nest_R [5′-TTACAAAGTCTAACGACGTGCAG-3′]), 250 μM each dNTP, 2.5 mM $MgCl_2$, and 2.5 U *Taq* polymerase (FirePol DNA polymerase I; Solis BioDyne). Amplifications were performed under the following conditions: 94°C for 5 min; 35 cycles of 94°C for 30 s, 57°C for 90 s, and 72°C for 90 s; and a final extension at 72°C for 10 min (681 bp).

PCR products were purified, using a polyacrylamide gel (Bio-Gel P-100; Bio-Rad, Marnes-la-Coquette, France), by 96-well plate filtration (Millipore, St. Quentin en Yvelines, France). Sequencing reactions were performed using the ABI Prism BigDye Terminator cycle sequencing ready reaction kit and were run on a 3730xl genetic analyzer (Applied Biosystems, Courtaboeuf, France). Electrophoregrams were visualized and analyzed with CEQ 2000 Genetic Analysis System software (Beckman Coulter). Amino acid sequences were compared with the wild-type sequence (GenBank accession no. AY846633 for *Pmdhfr*) using the BioEdit Sequence Alignment Editor (16). The presence of single nucleotide polymorphisms (SNP) was confirmed by reads through both forward and reverse strands. The *Pmdhfr* gene polymorphisms identified among our isolates were compared to *dhfr* gene sequences of *P. malariae* available in GenBank.

Statistical analysis. Raw data were input into Microsoft Excel 2007 software and were checked and analyzed using MedCalc software, version 9.1.0.1 (MedCalc Software, Mariakerke, Belgium). Continuous variables were compared by using the independent-sample t test. Sequence polymorphism analysis, including the calculation of haplotype diversity (h) and nucleotide diversity (π), was performed using DnaSP software, version 5.10.01 (33). Departures from selective neutrality were assessed by the ratio of nonsynonymous to synonymous substitutions (dN/dS ratio) in the *P. malariae dhfr* gene. Significance (probability of rejecting the null hypothesis of strict neutrality [$dN = dS$]) was determined by using the two-tailed Z-test (bootstrap method with 10,000 replicates using the Nei-Gojobori method) as implemented in MEGA, version 5.0.5 (36). In all tests performed, P values of <0.05 were considered statistically significant at the 5% level.

Ethical approval. Ethical clearance for the samples used in this study was obtained from the Ethics Committee of the Cambodian Ministry of Health and the Ethics Committee of the Ministry of Health of Madagascar (007/SANPF/2007; registration number ISRCTN36517335). Informed written consent was provided by all patients or their parents/guardians before inclusion in the study.

Nucleotide sequence accession numbers. The sequences for the new haplotypes of the *Pmdhfr* gene identified here have been deposited in GenBank under accession numbers JN038065 to JN038071.

RESULTS

***P. malariae* dihydrofolate reductase polymorphisms.** A 633-bp fragment (spanning the region from codon 15 to codon 225 in the *Pmdhfr* gene) was sequenced from 123 clinical *P. malariae* isolates: 26 from Cambodia (including 5 *P. falciparum*-*P. malariae* and 3 *P. vivax*-*P. malariae* mixed infections), 53 from Madagascar (including 13 mixed infections with *P. falciparum* and *P. malariae* and 1 with *P. vivax* and *P. malariae*), and 44 from various African countries (19 from Côte d'Ivoire, 17 from Cameroon, 4 from Comoros, 2 from Mali, 1 from Gabon, and 1 from Togo) (including 9 *P. falciparum*-*P. malariae* mixed infections).

A total of 623 monomorphic and 10 polymorphic sites were found. Polymorphic sites included those with 2 synonymous substitutions (V60V [GT**A** to GT**G**]; S114N [A**G**C to A**A**T] [a nonsynonymous substitution was also found at this site]) and 8 nonsynonymous substitutions. The 2 silent mutations were observed only once (in one Cambodian isolate and one Malagasy sample). Of the 8 sites with nonsynonymous substitutions, all were dimorphic: K55E, S58R, S59A, S114N, F168S, N194S, D207G, and T221A. Most of the nonsynonymous substitutions (6/8 [75%]) were nonconservative, with changes of the physicochemical family of the amino acid. The substitutions observed at codons 55, 58, 168, 194, 207, and 221 were found once (0.81%), while those affecting codon 114 were observed in three Cambodian isolates (2.4%) and those affecting codon 59 in four Malagasy samples (3.2%). The polymorphisms in the *Plasmodium malariae dhfr* gene (including those previously described by Tanomsing et al. [37]) are detailed in Fig. 1.

By following the numbering of haplotypes used by Tanomsing et al. (37), the polymorphisms observed in our samples were arranged into nine different haplotypes (H4, H5, and H8 to H14) and eight *Plasmodium malariae* DHFR proteins (H4, H5/H12, H8

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Polymorphisms in *P. malariae dhfr* gene																	
		Nucleotide*	66	142	150	163	172	175	340	341	341/342	503	510	581	620	661	Prevalence of haplotype
			CAT	AAA	AAT	AAA	AGC	TCA	AGC			TTT	ATT	AAT	GAT	ACT	
			CAG	GAA	AAG	GAA	CGC	GCA	GGC	AAC	AAT	TCT	ATG	AGT	GGT	GCT	
		Amino Acid	22	48	50	55	58	59	114			168	170	194	207	221	
			Q/H	K/E	N/K	K/E	S/R	S/A	S/G	S/N		F/S	I/M	N/S	D/G	T/A	
Location and number of the samples** from: Present study	GenBank	Haplotype															
	VN (2), GB (1)	1	■														0%
	VN (1)	2	■						■								0%
	GB (1)	3	■	■													0%
CAM (23), MAD (45), AFR (44)	TH (18), LAO (4), PNG (2), VN (1), GB (1)	4															91.0%
CAM (2)	TH (2)	5								■							1.6%
	LAO (1)	6			■												0%
	VN (1)	7											■				0%
MAD (1)		8				■											0.8%
MAD (1)		9					■					■					0.8%
MAD (3)		10						■									2.6%
MAD (1)		11						■							■		0.8%
CAM (1)		12									■						0.8%
MAD (1)		13												■			0.8%
MAD (1)		14														■	0.8%
Prevalence of mutation			0%	0%	0%	0.8%	0.8%	3.2%	0%	1.6%	0.8%	0.8%	0%	0.8%	0.8%	0.8%	

FIG 1 *dhfr* polymorphisms identified in 123 *Plasmodium malariae* isolates from Cambodia (CAM), Madagascar (MAD), and various African countries (AFR) (including Comoros, Côte d'Ivoire, Guinea, Cameroon, Mali, Gabon, and Togo) or described previously by Tanomsing et al. (37) and available in GenBank. *, nucleotide and amino acid numbers are given according to the wild-type sequence for *Pmdhfr* (GenBank accession no. AY846633); **, sample locations (with the numbers of isolates given in parentheses) are abbreviated as follows: VN, Vietnam; GB, Guinea-Bissau; TH, Thailand; LAO, Lao People's Democratic Republic; PNG, Papua New Guinea.

to H11, H13, and H14) (Fig. 1). Haplotype H4 was the most prevalent (112/123 [91.0%]) and was considered the wild-type sequence (see Discussion). The other eight haplotypes were classified as either single mutant (H10 [S59A], H5 and H12 [S114N], H14 [T221A], H13 [N194S], and H8 [K55S]) or double mutant (H11 [S59A D207S] and H9 [S58R F168S]) types. By comparison of the polymorphisms identified in our sample population ($n = 123$) to sequences available in GenBank ($n = 35$), seven new mutations (K55E, S58R, S59A, F168S, N194S, D207G, and T221A) and seven new haplotypes (H8 to H13), leading to six new DHFR proteins, were observed (Fig. 1).

All new mutations and haplotypes observed in our sample set were recognized as exclusive to Madagascar (Fig. 1), except for the double mutations at nucleotides 341 and 342 (S114N) found in one Cambodian isolate. This isolate, classified as a new haplotype (H12), leads to a DHFR protein described previously in Thailand (H5). Only three isolates from Cambodia were classified as mutant type (S114N), while all isolates from African countries were classified as wild type.

The molecular diversity indices and neutrality tests for each *P. malariae* population are given in Table 1. The overall nucleotide diversity and the mean haplotype diversity in our sample population were 0.0004 and 0.171, respectively. Haplotype and nucleotide diversities were significantly higher in the Malagasy than in the Cambodian *P. malariae* population (P, 0.005 and 0.001, respectively) and significantly higher among Cambodian *P. malariae* sequences than among African *P. malariae* sequences (P, <0.0001). Analyses of the dN/dS ratio showed a negative ratio for Cambodian samples (-0.94), indicative of purifying selection, while Malagasy samples had a ratio greater than 1 (1.02), suggesting positive selection. For African samples, in which no synonymous mutations were detected, the dN/dS ratio was zero. However, in all three populations, the probability of rejecting the null hypothesis of strict neutrality ($dN = dS$) was nonsignificant.

TABLE 1 *dhfr* polymorphisms identified in 123 *Plasmodium malariae* isolates from Cambodia, Madagascar, and various African countries

Characteristic	Value for: Cambodia	Madagascar	African countries	All sites
Sample size	26	53	44	123
No. of nucleotide sites observed	633	633	633	633
Monomorphic	631	625	633	623
Polymorphic	2	8	0	10
No. of changes				
Synonymous	1	1	0	2
Nonsynonymous	1	7	0	8
No. of haplotypes	3	7	1	9
Haplotype diversity (h) (SD)	0.218 (0.103)	0.279 (0.08)	0 (0)	0.171 (0.046)
Nucleotide diversity (π) (SD)	0.00048 (0.00024)	0.00067 (0.00024)	0 (0)	0.0004 (0.00013)

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Plasmodium sp.		Amino acid codon																
P. falciparum	Position	14	**16**	23	49	**50**	**51**	56	58	**59**	60	62	**108**	162	**164**	188	201	215
	Wild-type	I	**A**	K	K	**C**	**N**	K	F	**C**	A	T	**S**	F	**I**	N	D	T
	Mutant-type	-	**V**	-	-	**R**	**I**	-	-	**R**	-	-	**N/T**	-	**L**	-	-	-
P. vivax	Position	**13**	15	22	48	49	50	55	**57**	**58**	59	**61**	**117**	171	**173**	197	210	224
	Wild-type	**I**	A	T	K	C	N	K	**F**	**S**	S	**T**	**S**	F	**I**	N	D	N
	Mutant-type	**L**	-	-	-	-	-	-	**L/T**	**R**	-	**M**	**N**	-	**L**	-	-	-
P. malariae	Position		15	*22*	*48*	49	***50***	**55**	57	**58**	**59**	61	**114**	**168**	***170***	*194*	*207*	*221*
	Wild-type		A	*Q*	*K*	S	***N***	**K**	F	**S**	**S**	T	**S**	**F**	***I***	*N*	*D*	*T*
	Mutant-type		-	*H*	*E*	-	***K***	**E**	-	**R**	**A**	-	**N**	**S**	***M***	*S*	*G*	*A*

FIG 2 Correspondence between polymorphisms in the *P. malariae dhfr* gene and equivalent residues known to be related to pyrimethamine resistance in the *P. falciparum* and *P. vivax dhfr* genes. For *P. falciparum* and *P. vivax*, codons resulting from nonsynonymous mutations related to pyrimethamine resistance are indicated by boldface and shading. For *P. malariae*, codons likely related to pyrimethamine resistance are indicated by boldface and shading, while those close to SNP involved in *P. falciparum* or *P. vivax* pyrimethamine resistance are shown in boldface only. *P. malariae* codons in italics correspond to mutations described previously by Tanomsing et al. (37).

The correspondences between polymorphisms in the *P. malariae dhfr* gene and equivalent residues already known to be related to pyrimethamine resistance in the *P. falciparum* and *P. vivax dhfr* genes are presented in Fig. 2. Among the eight mutations described in the *P. malariae dhfr* gene, two were likely related to pyrimethamine resistance: S58R (H9; corresponding to C59R in *P. falciparum* and S58R in *P. vivax*; observed in one Malagasy sample) and S114N (H5 and H12; corresponding to S108N in *P. falciparum* and S117N in *P. vivax*; observed in three Cambodian samples). Three other mutations close to SNP involved in pyrimethamine resistance were also found: K55E (H8; observed in one Malagasy sample), S59A (H10 and H11; observed in four Malagasy samples), and F168S (H9; in association with S58R, observed in one Malagasy sample).

DISCUSSION

Although the distribution of *P. malariae* infection is reported as patchy, it has been observed in all major regions of the world where malaria is endemic (18). More common in sub-Saharan Africa and the southwest Pacific (3, 5, 6, 12, 38), *P. malariae* is also detected in Asia (7, 11, 13, 14, 21), the Middle East (1), and South and Central America (34, 39). Responsible for quartan malaria, *P. malariae* is usually considered a benign pathogen compared to human sympatric *P. falciparum* and *P. vivax*. Severe clinical symptoms are rarely associated with the acute infection, except in cases of chronic nephropathy. To date, data from drug susceptibility studies on this species are scare, while treatments for *P. falciparum* and *P. vivax* malaria are often used in a context of mixed infections with *P. malariae*. *In vitro* assays of *P. malariae* are not currently available, and therapeutic efficacy studies are very difficult to conduct. Molecular investigations focused on putative molecular markers remain the only means of assessing the impact of antimalarial drugs on this species, especially by extrapolating to what we have already observed for *P. falciparum* or *P. vivax* parasites.

In this study, we have analyzed a total of 123 sequences from clinical *P. malariae* isolates from Cambodia, Madagascar, and various African countries. Among these, 10 polymorphic sites, including 2 sites with synonymous substitutions and 8 with nonsynonymous substitutions (codons 55, 58, 59, 114, 168, 194, 207, and 221), were found. One of the most interesting results was that glutamine (Q) was the most prevalent amino acid at position 22. This amino acid was previously considered mutant type (H22Q) by Tanomsing et al., who had used the *P. brasilianum dhfr* sequence as the wild-type reference sequence, despite the fact that more than 85% (30/35) of their sequences had a glutamine at this position (37). Their main rationale for using *P. brasilianum* as the wild-type sequence was that given the widespread use of SP and the apparent ease in which *P. malariae* acquires resistance to pyrimethamine (42, 43), they suspected that all the sequences obtained from *P. malariae* isolates could already carry mutations, unlike the laboratory-maintained *P. brasilianum* strain. However, several indications seem to show otherwise: (i) the high prevalence of the haplotype carrying a glutamine at position 22, (ii) the very high susceptibility of this haplotype to pyrimethamine by use of the classical *in vitro* assay (50% inhibitory concentration [IC_{50}], <7 ng/ml) (37), (iii) the lack of equivalent residues in *Pfdhfr* and *Pvdhfr* previously associated with pyrimethamine resistance, (iv) the absence of a significant reduction in the energy of binding between pyrimethamine and the modeled PmDHFR enzyme observed in computational analysis (8), and (v) the difference in amino acid composition at codon 206 between sequences of *Plasmodium brasilianum* (valine) *and Plasmodium malariae* (isoleucine). All these indications lead us to suggest that the *Pmdhfr* wild-type sequence carries a glutamine at residue 22.

Compared to previous data, seven new mutations (at positions 55, 58, 59, 168, 194, 207, and 221) were observed exclusively in Malagasy samples, while the African isolates presented no mutations and the Cambodian samples presented only one mutation (S114N), already found in Thai samples. In total, nine haplotypes were found among our samples: two were shared with the sequences of Tanomsing et al. (37) (the wild-type haplotype and the S114N haplotype shared by Cambodian and Thai *P. malariae* sequences), and seven (exclusively from Malagasy samples) have never been described previously. However, the prevalences of these new haplotypes were very low (from 0.80% to 3.25%) compared to that of the wild type (91.05%). Among the new haplotypes, two were classified as double mutant type (S59A D207S and S58R F168S), while the others were classified as single mutant type (S59A, S114N, T221A, N194S, and K55S). Of the four mutations (leading to changes in residues 50, 58, 114, and 170) previously found to be associated with pyrimethamine resistance in *P. falciparum* and *P. vivax* (Fig. 2), only two were observed in our samples: S58R in one Malagasy sample (0.80%) and S114N in three Cam-

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bodian isolates (2.40%). We did not find mutations at position 50 (observed in one Lao People's Democratic Republic sample) or 170 (observed in one Vietnamese sample) in our set of samples. Recent data from *in silico* experiments on binding between modeled *Pmdhfr* mutant sequences and antifolates revealed that certain residues (I13, L45, D53, S117, and I170) appear to play important roles in binding with pyrimethamine (8). Among the mutations (N50K, S58R, S114N, I170M) found in field isolates from their previous work (37) that correlate with those seen in PfDHFR and PvDHFR, the authors showed that only the *Pmdhfr* I170M mutant type was associated with a significant reduction in docking energy for pyrimethamine, suggesting differences between species in terms of the ability of the DHFR enzyme to tolerate mutations. Three other mutations close to SNP involved in pyrimethamine resistance (K55E, S59A, and F168S) were also found in Malagasy samples. These mutations were not directly studied by Choowongkomon et al. (8) in their experiments, but they could have indirect implications for ligand binding by DHFR enzymes. For example, we cannot exclude the possibility that the S59A mutation was selected by antifolate drug pressure, since it is closely related to the S58R mutation, or that the mutation observed at codon F168S, which is closely related to I170M, would be an important target for the selection pressure of pyrimethamine resistance against PmDHFR. Further studies involving heterologous expression of the PmDHFR mutants found in our study would be useful in addressing this issue.

Comparing *P. malariae dhfr* sequences in Cambodian, Malagasy, and African populations, we observed greater haplotype and nucleotide diversity among the Malagasy *P. malariae* population. These data could be explained partly by the fact that the prevalence of *P. malariae* infections is about 10-fold higher in Madagascar than in Cambodia (4, 20). In both populations, wild-type *P. malariae* isolates were largely predominant (around 85%). In the Cambodian population, only one mutant type haplotype (114N) that correlates with *P. falciparum* or *P. vivax* mutant type haplotypes was observed, suggesting that this allele was selected when SP was used as an antimalarial in the 1980s (19). In Madagascar, *P. malariae* DHFR mutant type alleles are more polymorphic, a pattern likely related to the pyrimethamine pressure exerted with the increased use of SP since the implementation of intermittent preventive treatment (IPT) for pregnant women (28).

In conclusion, polymorphism in the *Plasmodium malariae dhfr* gene appears to be low compared to that in its sympatric *Plasmodium* species. However, according to our data from Cambodian and Malagasy isolates, local epidemiology and SP drug pressure history influence the selection of DHFR mutations, as is the case for *P. falciparum* and *P. vivax*. Additional studies comparing *dhfr* sequences in *Plasmodium* species from the same population or from patients with mixed infections would be worthwhile.

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Manuscrit en préparation 1

INTRODUCTION

Until now, five malaria *Plasmodium* species infected humans including *Plasmodium falciparum*, *P. vivax, Plamosium malariae, Plasmodium knowlesi*, *Plasmodium ovale* (*Plasmodium ovale curtisi,* classic type, *PoC* and *Plasmodium ovale wallikeri,* variant type*, PoW)* (Fuehrer et al. 2012, Sutherland et al. 2010) have been described. Most studies are focused on *Plasmodium falciparum* and *Plasmodium vivax* identification, the infrequency of *Plasmodium malariae* and *Plasmodium ovale* would be challenging (Kawamoto et al. 1999). Moreover, the identification of these species through to light microscopy was poor detection due to their confusing of morphological similarity between *Plasmodium ovale* and *Plasmodium vivax* (Collins and Jeffery 2005). A part from the published data, which reported in (Incardona et al. 2005), described the *Plasmodium ovale* detection among the population in North-eastern village of Cambodia by using a species-specific polymerase chain reaction of small subunit ribosomal RNA (*ssurRNA*) gene, then never study was carried on. Up to date, we do not know which subspecies of *Plasmodium ovale* were presented in Cambodia.

In this context, we conducted a research as part of a collaboration between National Centre for Parasitology Entomology and Malaria Control, Malaria Molecular Epidemiology Unit from Pasteur Institute of Cambodia and the National Reference Centre for Malaria (NRCM, Paris, France) aims 1) to assess the worldwide polymorphism of *Plasmodium ovale dhfr* in samples collected from Africa and Asia, areas with different histories of SP use by doing a comparative polymorphism in *dhfr* gene in sympatric *Plasmodium species* (*P. falciparum, P. Vivax*, *P. Malariae, Plasmodium ovale curtisi and Plasmodium ovale wallikeri*) in Cambodian and other African isolates and (2) the identification of subspecies of *Plasmodium ovale* among Cambodian parasite population.

MATERIALS AND METHODS

Samples collections

P. ovale isolates were collected in Cambodia and other countries in Africa. The Cambodian specimens were taken by venipuncture bloods and dried blood spots of symptomatic or asymptomatic cases in different areas of previous studies in 2001, 2004, 2007, 2010 and 2012, stored in the samples bank in the Malaria Molecular Epidemiology Unit at Pasteur Institute in Cambodia. The samples from various countries in Africa (Burkina (BUR), Cameroun (CM), Comoros (COM), Congo (CON), Côte d'Ivoire (CI), Guinea (GUI), Mali (MA), Republic of Central Africa (CAR), Senegal (SEN), and Zaire (DRC)) which were obtained from malaria-infected travellers in diverse countries in Africa returned back to France between 2001-2009, were supplied by the National Reference Centre for Malaria (NRCM), Paris France.

DNA extraction

The genomic DNA was extracted from 200ul of blood isolates or one punch of dried blood spot by using a DNA blood kit (Qiagen, Germany) or Instagen matrix (Bio-Rad, Marnes la Coquette, France) respectively according to manufacturer's instructions. These DNA were stored at -20^{0}C until use.

A total of 83 of *Plasmodium ovale* isolates in this study (11 from Cambodia (CAM), 72 from African isolates (1, 16, 6, 5, 32, 1, 4, 2, 1, 4 from BUR, CM, COM, CON, CI, GUI, MA, CAR, SEN, DRC respectively)) were amplified and sequenced for *dihydrofolate reductase* gene.

Amplification and sequencing

To confirm the presence of *Plasmodium ovale*, a total of 6000 accumulative samples were determined for *Plasmodium spp.* infection by Nested PCR CytB for Cambodian isolates as described in (Steenkeste et al. 2009) or by real time PCR for African individuals as previously published in (Brega et al. 2004).

The *dihydrofolate reductase* gene was the target of the amplification. The Nested PCR tools were used to get more amplicon. The degenerative primers were designed by using Primer3 to amplify this target. The primary PCR was established in 25µl of final volume with 0.25µM of each primer (Podhfr_PCR_F, 5'- mgabgtwttcgayatatacgc -3', Podhfr_PCR_R, 5'- crccatttcgatgatcctt -3') (Sigma-Aldrich®, Singapore), 0.25mM of each deoxynucleoside triphosphate (dNTP) (Solis Biodyne), 1x of reaction Buffer, 2.5mM of MgCl2, 1.25U Taq polymerase (FirePol® DNA Polymerase, Solis Biodyne). This first round of this PCR was done with the conditions as below: 94^{0}C for 5 min, then 40 cycles of 94^{0}C for 30 sec, 52^{0}C for 30 sec, and 72^{0}C for 1min 30 sec. This amplicon was attained by final prolongation at 72^{0}C for 10 min to reach the fragment of 858bp. An artificial sequenced tail (in bold) was added to the nested primers sequence for doing sequencing by Sanger technique. The Nested PCR was performed in 50µl of final volume in 0.4µM of each primer (Podhfr_Nested_F, 5'-**ctcgaggaattcggatcc**cgtgctgcaargtctccaa -3', Podhfr_Nested_R, 5'-**tctagaaagcttggatcc**ttccgtgrtgagaagaagca -3') (Sigma-Aldrich®, Singapore), 0.25mM of each deoxynucleoside triphosphate (dNTP) (Solis Biodyne), 1x of reaction Buffer, 2.5mM of MgCl2, 2.5U Taq polymerase (FirePol® DNA Polymerase, Solis Biodyne). This second round of this PCR was done such as the conditions: 94^{0}C for 5 min, then 40 cycles of 94^{0}C for 30 sec, 57^{0}C for 30 sec, and 72^{0}C for 1min 30 sec, then followed by a final extension at 72^{0}C for 10 min to obtain the fragment of 810bp. For all these assays 5ul of the template was used to initiate the amplification reactions. PCR products were purified, using polyacrylamide P-100 Gel (Bio-Gel P-100, BioRad®, Marnes-la-Coquette, France), by 96-well plate filtration (Millipore®, St. Quentin en Yvelines, France). Sequencing reactions were performed using ABI PRISM BigDye Terminator cycle sequencing ready reactions kit and run on a 3730 xl Genetic Analyzer (Applied Biosystems, Courtaboeuf, France). Electrophoregrams were visualized and analyzed with CEQ™ 2000 Genetic Analysis System software (Beckman Coulter™). Amino-acid sequences were compared with wild-type sequences (Gen- Bank accession no. XM_001351443 for *Pfdhfr*, X98123 for *Pvdhfr* and AY846633 for *Pmdhfr*), using BioEdit Sequence Alignment Editor Software (Kawamoto et al. 1999). Parasites with mixed alleles (both wild-type and mutant alleles present) were considered to be mutant. The *Podhfr* gene

polymorphisms identified from the isolates in this study were aligned to all *Podhfr* gene sequences which are available in GenBank.

RESULTS AND DISCUSSION

Plasmodium ovale dihydrofolate reductase polymorphisms

By using CEQ2000, from 810 bp of *Podhfr* amplicon, we analyzed 738bp (AA 30-AA275) which was sequenced from 83 individuals *Plasmodium ovale* that contain 11 Cambodian isolates and 72 other French cases who came back from various African countries (1, 16, 6, 5, 32, 1, 4, 2, 1, 4 from BUR, CM, COM, CON, CI, GUI, MA, CAR, SEN, DRC respectively). The polymorphisms in *Plasmodium ovale dhfr gene* (including those 20 cases previously described by Sutherland et al., (Sutherland et al. 2010)), are detailed in Table 1, were classified into 26 different haplotypes namely from A to Z.

Polymorphisms in Plasmodium ovale curtisi

6 new haplotypes were introduced among the parasite population of *Plasmodium ovale curtisi*. Haplotype K (39 individuals) was the most predominate in this research which constitute 17, 9, 4, 2, 2, 2, 1, 1, 1 isolates from CI, CAM, CM, COM, CON, MA, SEN, GUI, CAR successively. Furthermore, haplotype L was present in 4 samples from CM. 2 Cambodian isolates have been also determined for 2 unlike haplotype J and Z. Then followed by one sample of each from CON, and CI were named in haplotype M and N. On the other hand, 4 samples of GB previously described in (Tirakarn et al. 2012) could be distinguished in 4 different haplotypes O (EU266609) that contains double mutation N70**D** and I96**M**, P(EU266611), V(EU266608), Y(EU266610) which constitutes a double mutation C166**R** and D204**G**. It is note that a mutation at I212**V** and 2 other inverse alleles of *Plasmodium ovale wallikeri* at AA 263 and 265 were detected in haplotype P. Moreover, 2 STP isolates were differentiated into two haplotypes W (EU266607) which has a non-synonymous mutation at P118**L** and a silent mutation T250**T**, X (EU266606).

In this report, 6 news single nucleotide polymorphisms (*) were inserted into the study reported by (Sutherland et al. 2010, Tirakarn et al. 2012). These polymorphic sites contain 3 synonymous substitutions (G38G” GGT⇒ GG**C**”, S112**S**”TCT⇒TC**C**”, G223G “GGA⇒GG**G**”) and 3 other non-synonymous substitutions (K48**R** “AAG⇒A**G**G”, V200**I** “GTT⇒**A**TT”, T251**I** “ACT⇒A**T**T”). The silent mutation at codon G38**G** were observed in one isolate from CI; S112**S** was found once from GB, haplotype V(EU266608); both SNP were considered as *Plasmodium ovale curtisi* , and G223**G** was discovered in one Thai isolate (EU266616) that was treated as *Plasmodium ovale wallikeri*. The non-synonymous substitutions K48**R**, V200**I** and T251**I**, which were only proceeded as *Plasmodium ovale curtisi*, were seen once from CON (haplotype M) and in two different Cambodian isolates (haplotype J and Z respectively). The polymorphism site at AA140 of *Plasmodium ovale wallikeri* was detected in almost *Plasmodium ovale curtisi* in this project.

Polymorphisms in Plasmodium ovale wallikeri

6 other new haplotypes, which were cited as haplotype B to G, were innovated within the parasite population of *Plasmodium ovale wallikeri*. There were 5 samples from CON=1, CI=2, COM=1, DRC=1 have common haplotype with 7 Thai isolates(EU266601, EU266602, EU266612, EU266614, EU26615, EU266618, GQ250090,), one Vietnamese sample (EU1026932), and 1 PNG (EU266603) which were reported in (Sutherland et al. 2010, Tirakarn et al. 2012). 26 individuals (CI=10, DRC=3, CM=7, COM=3, CON=1, MA=1, CAR=1) in this study were classified in haplotype B which has two microsatellite TATA. 2 other haplotypes, C that contains microsatellite TATATA and G which has microsatellite TATA and has the allele likely as *Plasmodium ovale curtisi* at 31Y, were shown in 2 different samples from CI. Followed by haplotype D that constitutes microsatellite TATATATA, E and F, which hold the common allele as *Plasmodium ovale curtisi* at 33N, but it did not have the same microsatellite (E has TATA, F has TA), were introduced in once of each from BUR, CM, MA respectively. According to the previous study, double mutations at I39**V** and Y56**C** and microsatellite TATA were seen in one sample from STP, haplotype Q (EU266605), a mutation at Y164**H** and one microsatellite TA was shown in one Thai individual, haplotype R (EU266613), double mutation (non-synonymous C178**Y** and synonymous G223**G**) and one TA was found in one Thai case, haplotype S (EU266616), a polymorphism site at I184**M** and one TATA was exposed in one isolate from BEN, haplotype T (EU266604), an inverse allele at 209 was cited in one Thai isolate, haplotype U (EU266617).

SNP in *Plasmodium dihydrofolate reductase* gene are likely associated to pyrimethamine resistance in *Plasmodium falciparum* (A16**V**, C50**R**, N51**I**, C59**R**, S108**T/N**, I164**L**), *Plasmodium vivax* (I13**L**, F57**L/T**, S58**R**, T61**M**, S117**N**, I173**L**), *Plasmodium malariae* (S58**R**, S114**N**, I170**M**).
Only four isolates from Cameroon were classified as mutant type (S58**R**) of *Plasmodium ovale curtisi* which correspond to C59**R** (Peterson et al. 1990, Plowe et al. 1998) in *Plasmodium falciparum*, S58**R** in *Plasmodium vivax (Barnadas et al. 2008, Choowongkomon et al. 2010, Tirakarn et al. 2012)*, S58**R** in *Plasmodium malariae* (Choowongkomon et al. 2010, Khim et al. 2012, Tanomsing et al. 2007), while other samples in this study including the 20 individuals from GenBank were cited as wild type (Table 2).

Geographical distribution of Plasmodium ovale sub species by using dihydrofolate reductase gene (Figure 1)

In this study, *Plasmodium ovale curtisi* was only discovered in Cambodian isolates (11/11) which is strange compared to the other cases from the countries in the region that are mostly observed for *Plasmodium ovale wallikeri*. According to the previous studies, the *Plasmodium ovale* variant type (*Plasmodium ovale wallikeri*) is principally determined for Thai isolates (10/10) (Sutherland et al. 2010, Tirakarn et al. 2012). Moreover, *Plasmodium ovale wallikeri* is present in one sample from Papua New Guinea, another one of Vietnamese isolate (Sutherland et al. 2010).

Regarding the African isolates, the samples from Guinea-Bissau, Guinea, and Senegal (4, 1, 1 respectively) were typically defined only *Plasmodium ovale curtisi* (classical type). On the other

hand, *Plasmodium ovale wallikeri* have been observed in all samples from Zaire, Benin, and Burkina (4, 1, 1 respectively). Furthermore, both sub species were found in equally among parasite population from Cameroon (8PoC, 8PoW), Mali (2PoC, 2PoW) and Republic of Central Africa (1PoC, 1PoW). In addition, the classical and variant types of *Plasmodium ovale* were discovered within the isolates from Comoros (2 PoC, 4 PoW), Congo (3 PoC, 2 PoW), Côte d'Ivoire (18 PoC, 14 PoW), Sao Tome and Principe (2 PoC, 1 PoW).

In this research, 56.63% (47 isolates) of *Plasmodium ovale curtisi* and 43.37% (36 samples) of *Plasmodium ovale wallikeri* were characterized by studying the *dihydrofolate reductase gene*.

CONCLUSION

Even though the limited number of 11 Cambodian isolates could be identified for *Plasmodium ovale curtisi* (classical type). It is the first evidence that none of the variant type of *Plasmodium ovale* was present among Cambodian parasite population which is dissimilar to the other individuals around our country.

Table 1. ***Podhfr*** **polymorphisms identified in Cambodian and other isolates**

Table 1: *Podhfr* polymorphisms identified in Cambodian, and Other isolates

Sample locations** Present study (N)	GenBank	Haplotype																																							Prevalence of polymorphism
		Nucleotide	92	98	102	118*	115	143*	146	156	167	172	208/21	288	303	336*	353/354	381	396	420	490	496	533	552	559	598*	611/612	619	625	634	669*	672	681	744	750	752*		801	812	817/819	
		PoC	TAT	AAT	TCT	GGT	ATC	AAG	TAT	GTA	TAT	AGT	AAT	ATA	AAG	TCT	CCC	AGG	TTA	GAC	TAC	TGT	TGT	ATT	GTA	GTT	GAA	AAA	ACT	ATA	GGA	ACT	GAT	TCC	ACT	ACT	771/789	GGG	GAT	ACC	
		PoW	TTT	AGC	TCA	GGT	ATC	AAG	TGT	GTT	TAT	AGT	AAG	ATA	AAA	TCT	CCT	AGA	TTG	GAT	TAC	TGT	TGT	ATT	ATA	GTT	GAT	GAA	GCT	ATA	GGA	ACC	GAC	TCA	ACA	ACT		GGA	GCT	TCT	
		mut				GGC	GTC	AGG			TGT	CGT	GAT	ATG		TCC	CTC				CAC	CGT	TAT	ATG		ATT	GGA			GTA	GGG				ACC	ATT					
		AA	31	33	34	[illegible]	39	48*	49	52	56	58	70	96	101	117*	118	127	132	140	164	166	178	184	187	200*	204	207	208	212	223*	224	227	248	250	251*		259	268	265	
		PoC	Y	N	S	G	I	K	Y	V	Y	S	N	I	K	S	P	R	L	D	Y	C	C	I	V	V	E	K	T	I	G	T	D	S	T	T	252/252	G	D	T	
		PoW	F	S	S	G	I	K	C	V	Y	S	K	I	K	S	P	R	L	D	Y	C	C	I	I	V	D	E	A	I	G	T	D	S	T	T		G	A	S	
		mut				G	V	R			C	R	D	M		S	L				H	R	Y	M		I	G			V	G				T	I					
M (1)		10 J*																		■						■											no				1 1.0% PoC
CI (17), CAM (9), CM (4), OM(2), SEN (1), GUI (1), CON (2), MA (2), CAR (1)		11 K*																		■																	no				39 37.9% PoC
(4)		12 L*										■								■																	no				4 3.9% PoC
N (1)		13 M*						■												■																	no				1 1.0% PoC
(1)		14 N*				■														■																	no				1 1.0% PoC
	GB (1)	15 O											■	■						■																	no				1 1.0% PoC
	GB (1)	16 P																		■										■							no		■	■	1 1.0% PoC
	GB (1)	22 V														■				■																	no				1 1.0% PoC
	STP (1)	23 W															■			■															■		no				1 1.0% PoC
	STP (1)	24 X																																			no				1 1.0% PoC
	GB (1)	25 Y																		■		■					■										no				1 1.0% PoC
M (1)		26 Z*																		■																■	no				1 1.0% PoC
Prevalence of polymorphism in *Plasmodium ovale Curtisi*						1 1.0%	0.0%	1 1.0%	0.0%	0.0%	0.0%	4 3.9%	1 1.0%	1 1.0%	0.0%	1 1.0%	1 1.0%	0.0%	0.0%	52 50.5%	0.0%	1 1.0%	0.0%	0.0%	0.0%	1 1.0%	1 1.0%	0.0%	0.0%	1 1.0%	0.0%	0.0%	0.0%	0.0%	1 1.0%	1 1.0%	0.0%	0.0%	1 1.0%	1 1.0%	53 51.5% PoC
ON(1), CI (2), COM(1), DRC(1)	TH (7), VN (1), PNG (1)	1 A																																			ACA GCT				14 13.6% PoW
(10), DRC (3), CM (7), COM 3), CON (1), MA(1), CAR (1)		2 B																																			ACA GCT ACA GCT				26 25.2% PoW
CI (1)		3 C																																			ACA GCT ACA GCT				1 1.0% PoW
BUR(1)		4 D																																			ACA GCT ACA GCT				1 1.0% PoW
(1)		5 E		■																																	ACA GCT ACA GCT				1 1.0% PoW
(1)		6 F		■																																	ACA GCT				1 1.0% PoW
(1)		7 G	■																																		ACA GCT ACA GCT				1 1.0% PoW
	STP (1)	17 Q					■				■																										ACA GCT ACA GCT				1 1.0% PoW
	TH (1)	18 R																			■																ACA GCT				1 1.0% PoW
	TH (1)	19 S																					■								■						ACA GCT				1 1.0% PoW
	BEN (1)	20 T																						■													ACA GCT ACA GCT				1 1.0% PoW
	TH (1)	21 U																											■								ACA GCT				1 1.0% PoW
Prevalence of polymorphism in *Plasmodium ovale Wallikeri*			1 1.0%	2 1.9%			1 1.0%				1 1.0%										1 1.0%		1 1.0%	1 1.0%					1 1.0%		1 1.0%						18/30/1/1 17.5%/29.1%/1%/1%				50 48.5% PoW

Table 2. Sequence analysis of DHFR domain for *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale curtisi* and *Plasmodium wallikeri*

Table 2: Sequence analysis of dhfr

Amino acid codon

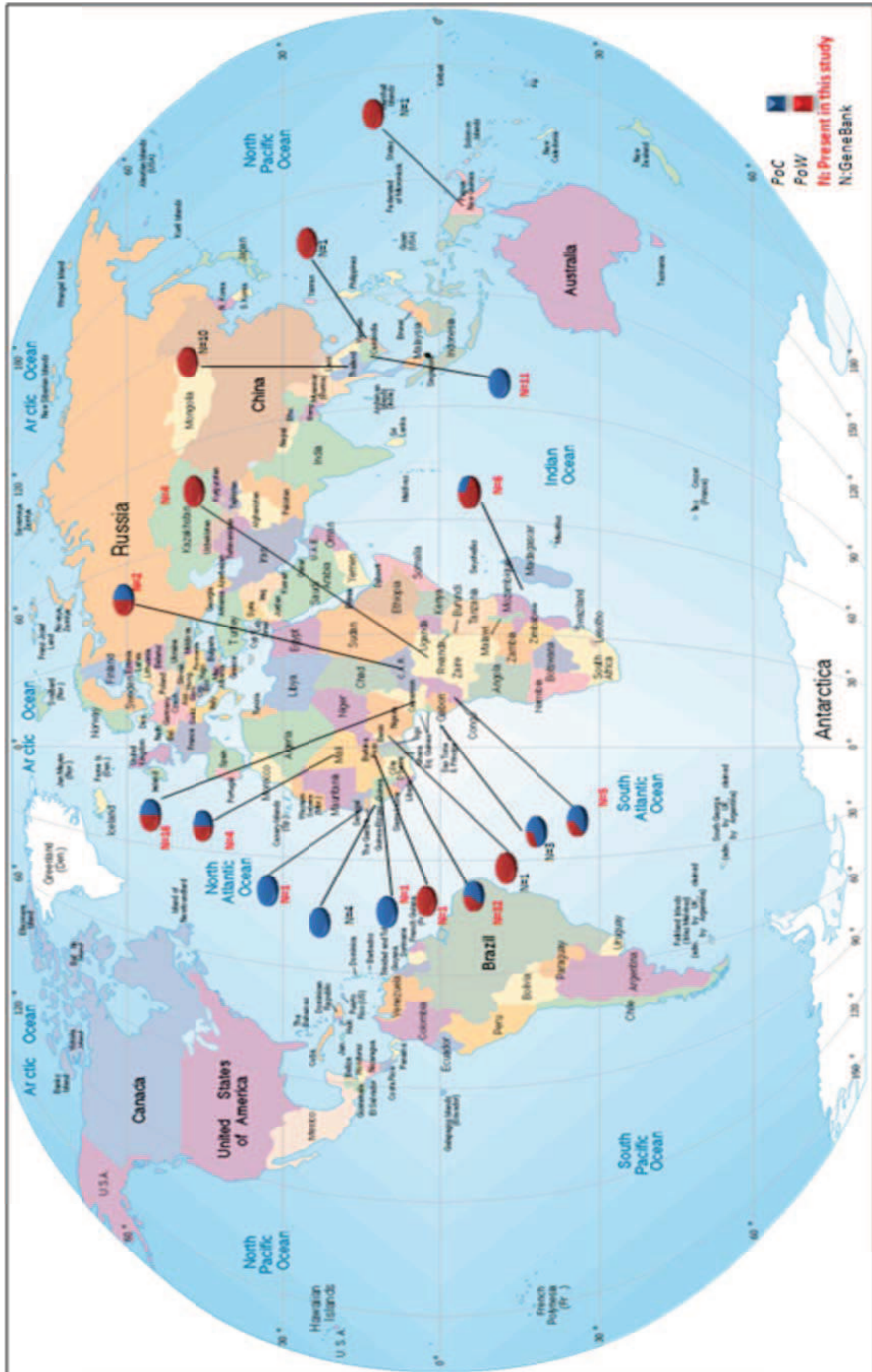
P. falciparum	Position	14	16	23	32	34	40	49	50	51	56	57	58	59	60	62	71	91	108	113	159	161	162	164	173	179	182	188	195	199	201	202	204	207	215	242	243	245	247
	Wild-type	I	A	K	F	N	L	K	C	N	K	Y	F	C	A	T	E	D	S	P	Y	C	F	I	F	I	I	N	V	E	N	E	E	I	T	G	E	K	N
	Mutant-type	-	V	-	-	-	-	-	R	I	-	-	-	R	-	-	-	-	N/T	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. vivax	Position	13	15	22	31	33	39	48	49	50	55	56	57	58	59	61	70	100	117	122	168	170	171	173	182	188	191	197	204	208	210	211	213	216	224	253	254	256	258
	Wild-type	I	A	T	F	P	L	K	C	N	K	Y	F	S	S	T	E	G	S	P	Y	C	C	I	C	I	I	N	V	E	D	E	Q	V	N	T	A	R	T
	Mutant-type	L	-	-	-	-	-	-	-	-	-	-	L/T	R	-	M	-	-	N	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. malariae	Position	13	15	22	31	33	39	48	49	50	55	56	57	58	59	61	70	97	114	119	165	167	168	170	179	185	188	194	201	205	207	208	210	213	221	252	253	255	257
	Wild-type		A	Q	F	T	L	K	S	N	K	Y	F	S	S	T	K	S	S	P	Y	C	F	I	C	I	I	N	V	E	D	E	V	I	T	D	K	S	T
	Mutant-type	-	-	H	-	-	-	-	-	-	E	-	-	R	A	-	-	-	N	-	-	-	S	M	-	-	-	S	-	-	G	-	-	-	A	-	-	-	-
									**	**				**					*																				
P. ovale Curtisi	Position	13	15	22	31	33	39	48	49	50	55	56	57	58	59	61	70	96	113	118	164	166	167	169	178	184	187	193	200	204	206	207	209	212	220	251	261	263	265
	Wild-type		A	E	Y	N	I	K	Y	N	S	Y	F	S	S	T	N	I	S	P	Y	C	F	I	C	I	V	N	V	E	D	K	T	I	S	T	A	D	T
	Mutant-type	-	-	-	-	-	-	R	-	-	-	-	-	R	-	-	D	M	-	L	-	R	-	-	-	-	-	-	I	G	-	-	-	V	-	I	-	A	S
P. ovale Wallikeri	Position	13	15	22	31	33	39	48	49	50	55	56	57	58	59	61	70	96	113	118	164	166	167	169	178	184	187	193	200	204	206	207	209	212	220	251	263	265	267
	Wild-type		A	E	F	S	I	K	C	N	S	Y	F	S	S	T	K	I	S	P	Y	C	F	I	C	I	I	N	V	D	D	E	A	I	S	T	A	A	S
	Mutant-type	-	-	-	Y	N	V	-	-	-	-	C	-	-	-	-	-	-	-	-	H	-	-	-	Y	M	-	-	-	-	-	-	-	-	-	-	-	-	-

SNP equivalent residus known to be associated to pyrimethamine resistance in *Plasmodium falciparum* and *Plasmodium vivax* dihydrofolate reductase gene

Mutation present in GeneBank

SNP present in this study

Figure 1. Geographical distribution of *P. ovale* subspecies by using *Podhfr* target



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O1.2. Impact de l'utilisation de la méfloquine sur les populations parasitaires de *Plasmodium vivax*

De nombreuses études ont montrés une association forte entre l'amplification du gène « multidrug resistance-1 » et la résistance à la méfloquine de *P. falciparum* soit à travers d'études cliniques soit à travers de données *in vitro*. Bien que les infections à *P. vivax* ne sont généralement pas traités avec la méfloquine, le diagnostic incorrect, la forte proportion d'infections mixtes non détectés ou l'apparition de relapses à *P. vivax* au cours du traitement d'une infection à *P. falciparum* font que fréquemment *P. vivax* est accidentellement exposé à la méfloquine.

Pour évaluer, l'impact de tel traitement non intentionnel sur les populations parasitaires de *P. vivax*, nous avons étudié la variation du nombre de copie du gène *Pvmdr-1* (PlasmoDB accession no. PVX_080100, NCBI reference sequence NC_009915.1) parmi 607 échantillons provenant de régions où la méfloquine a été ou n'a pas été utilisé ainsi que d'échantillons provenant de voyageurs utilisant parfois la méfloquine en prophylaxie.

Comme pour *P. falciparum*, nous avons observé que le nombre de copie du gène *Pvmdr-1* était également augmenté dans les zones où la méfloquine est régulièrement utilisée. Ces données, nous ont permis de démontrer que les traitements contre *P. falciparum* exercent bien des effets collatéraux, souvent méconnus sur les autres espèces et que les politiques mises en place en matière de traitement pour traiter les infections à *P. falciparum* doivent prendre en compte la présence des autres espèces.

Article 2: **Khim N**, Andrianaranjaka V, Popovici J, Kim S, Ratsimbasoa A, Benedet C, Barnadas C, Durand R, Thellier M, Legrand E, Musset L, Menegon M, Severini C, Nour BY, Tichit M, Bouchier C, Mercereau-Puijalon O, Ménard D (2014). Effects of Mefloquine Use on Plasmodium vivax Multidrug Resistance. Emerg Infect Dis. 20:1629-36.

Article 2

Effects of Mefloquine Use on *Plasmodium vivax* Multidrug Resistance

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Numerous studies have indicated a strong association between amplification of the *multidrug resistance-1* gene and in vivo and in vitro mefloquine resistance of *Plasmodium falciparum*. Although falciparum infection usually is not treated with mefloquine, incorrect diagnosis, high frequency of undetected mixed infections, or relapses of *P. vivax* infection triggered by *P. falciparum* infections expose non–*P. falciparum* parasites to mefloquine. To assess the consequences of such unintentional treatments on *P. vivax*, we studied variations in number of *Pvmdr-1* (PlasmoDB accession no. PVX_080100, NCBI reference sequence NC_009915.1) copies worldwide in 607 samples collected in areas with different histories of mefloquine use from residents and from travelers returning to France. Number of *Pvmdr-1* copies correlated with drug use history. Treatment against *P. falciparum* exerts substantial collateral pressure against sympatric *P. vivax*, jeopardizing future use of mefloquine against *P. vivax*. A drug policy is needed that takes into consideration all co-endemic species of malaria parasites.

Since World War II, antimalarial drugs have been intensively used to prevent or treat malaria (*1*). As observed with other antimicrobial agents, their use (or frequent misuse, when malaria diagnosis was based only on clinical symptoms without parasitologic confirmation) led to the emergence, selection, and spread of resistant parasites (*2*). This resistance became a global problem during the 1960s, when *Plasmodium falciparum* parasites developed resistance to chloroquine, the most widely used antimalarial drug at that time (*3*). In particular, resistant parasites that emerged in the Greater Mekong subregion of Asia later spread to Africa, triggering a dramatic increase in malaria and malaria-related deaths, particularly among children (*4*). During the 1980s, a similar scenario was observed with sulfadoxine-pyrimethamine (SP) when this association was recommended to replace chloroquine as first-line treatment in uncomplicated falciparum malaria (*5*,*6*). Since then, biological and molecular investigations using laboratory and field isolates have demonstrated that resistance of *P. falciparum* to antimalarial drugs is mediated by 2 major mechanisms: 1) a modification of the parasite target (i.e., mutations in *dihydrofolate reductase* [*dhfr*] or in *dihydropteroate synthetase* [*dhps*] genes) or 2) an increase of the efflux of the drug away from its site of action (i.e., mutations in the *chloroquine resistant transporter* gene or the *multidrug resistance-1* [*mdr-1*] gene or in an increased number of copies of the *mdr-1* gene). These molecular events have been intensively studied and are well known for *P. falciparum* but not for other *Plasmodium* species, mainly because of the ability to culture in vitro *P. falciparum* erythrocytic stages.

Our understanding of the molecular mechanisms of antimalarial drug resistance developed by *P. vivax* is less comprehensive. Although *Aotus* and *Saimiri* monkey models have provided useful information about *P. vivax* biology, most of the data have been gained through comparative studies investigating polymorphisms in orthologous genes encoding resistance to pyrimethamine (*dhfr* gene), sulfadoxine (*dhps* gene), or chloroquine (*chloroquine resistant transporter* or *mdr-1* genes). For instance, mutations in

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codons 57, 58, 61, 117, and 173 of *P. vivax* DHFR (corresponding to codons 51, 59, 108, and 164 of *P. falciparum* DHFR) are involved in resistance to pyrimethamine, although *P. vivax* infections are not usually treated directly with SP (*7*). This resistance was confirmed by heterologous expression studies, invalidating the common idea that *P. vivax* was "intrinsically resistant" to pyrimethamine (*8*), which suggests that the high frequency of mixed *P. falciparum*/*P. vivax* infections that are not detected by microscopy (*9–11*) or relapses of *P. vivax* infection after *P. falciparum* infections probably exposes *P. vivax* parasites to antimalarial drugs used to treat falciparum malaria infections, especially those with a long half-life, and selects *P. vivax* genetic traits conferring antimalarial drug resistance.

The impact of antimalarial drugs, especially those with long half-lives (such as mefloquine), on the sympatric *Plasmodium* species is not clearly understood. In areas where *P. falciparum* and *P. vivax* are co-endemic, such as South America and Southeast Asia, mefloquine has been widely used (alone in monotherapy or in combination with artemisinin derivatives) to treat uncomplicated falciparum malaria (*12*). In both areas, emergence of *P. falciparum* parasites resistant to mefloquine has been demonstrated from therapeutic efficacy studies (treatment failure) or in vitro testing (increased IC_{50} [half maximal inhibitory concentration]) and has been associated with the amplification of *P. falciparum mdr-1* (*Pfmdr-1*) gene (*13–16*). Recently, several studies performed on *P. vivax* samples collected in Southeast Asia (Thailand, Laos, Cambodia, and Myanmar) (*17–19*), South America (Brazil, Honduras) (*20*,*21*), and Africa (Mauritania) (*22*) have shown that *mdr-1* amplification does occur in *P. vivax*.

In this context, and to confirm the impact of the mefloquine drug pressure on *P. vivax* parasite populations, we used a real-time PCR to assess the number of *P. vivax mdr-1* (*Pvmdr-1*) gene copies to evaluate the worldwide distribution of *Pvmdr-1* amplification in samples collected from travelers with vivax malaria returning to France and from residents in areas with different histories of mefloquine use (French Guiana, Cambodia, Madagascar, and Sudan).

Materials and Methods

Sample Collection

P. vivax and *P. falciparum* samples from Madagascar were collected during 2006–2007 as part of the antimalarial drug resistance network, from symptomatic patients before treatment in 19 health centers located in areas of Madagascar with different epidemiologic patterns of malaria transmission: northern (Antsiranana, Antsohihy, Andapa), western (Mahajunga, Miandrivazo, Maevatanana, Morondava, Tsiroanomandidy, Ampasimpotsy), central (Saharevo, Moramanga), southern (Ihosy, Ejeda, Tolagnaro, Iakora, Ranostara, Toliara), and eastern (Farafangana, Toamasina). In Cambodia, *P. vivax* and *P. falciparum* isolates were obtained from symptomatic persons during 2010 in Pailin and Kratie Provinces. Other *P. vivax* samples were collected 1) from malaria-infected travelers returning to France after visiting Africa (Côte d'Ivoire, Ethiopia, Madagascar, and Mauritania), South America (Bolivia, Brazil, Colombia, French Guiana, Venezuela), and Asia (Bangladesh, Cambodia, India, Indonesia, Laos, Malaysia, Nepal, Pakistan, Sri Lanka) during 1997–2009 and were provided by the National Reference Center for Malaria (Paris, France); and 2) from symptomatic *P. vivax*–infected persons in French Guiana (2000–2003) or Sudan (2007).

DNA Extraction and PCR Detection of *P. falciparum* and *P. vivax*

We extracted parasite DNA from blood spots with Instagene Matrix (BioRad, Marnes-la-Coquette, France) or from whole blood samples using the phenol-chloroform method (*23*) or the QIAamp DNA Blood Mini Kit (QIAGEN, Courtaboeuf, France), according to the manufacturer's instructions. Molecular detection and identification of *Plasmodium* parasites were performed by using real-time PCR as described by Chou et al. (*24*).

Determination of the Number of *Pfmdr-1* Copies in Isolates from Cambodia and Malagasy

We measured number of *Pfmdr-1* copies using CFX96 real-time PCR (BioRad) relative to the single copy of the *β-tubulin* (used as a reference gene). Briefly, PCRs were conducted in 25-mL volumes in a 96-well plate containing 1X HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), 0.3 mM of each forward and reverse primer (*Pfmdr-1*, 5′-TGCATCTATAAAACGATCAGACAAA-3′ and 5′-TCGTGTGTTCCATGTGACTGT-3′; *β-tubulin*, 5′-TGATGTGCGCAAGTGATCC-3′ and 5′-TCCTTTGTGGACATTCTTCCTC-3′), and 4 mL of template DNA. Amplifications were performed under the following conditions: 94°C for 15 min, followed by 40 cycles of 94°C for 15 s, 58°C for 20 s, and 72°C for 20 s. The number of *Pfmdr-1* copies of each sample was measured in triplicate relative to a standard curve by using 4 standards of mixed plasmids cloned into TOPO cloning vector (Invitrogen, Saint Aubin, France): standard 1 (1:1 ratio of *Pfmdr-1* and β-tubulin), standard 2 (2:1 ratio of *Pfmdr-1* and *β-tubulin*), standard 3 (3:1 ratio of *Pfmdr-1* and *β-tubulin*) and standard 4 (4:1 ratio of *Pfmdr-1* and *β-tubulin*) and 2 parasite clonal lines used as controls, the 3D7 Africa line (1 copy of *Pfmdr-1*) and line Dd2 (3 copies of *Pfmdr-1*), by the ΔCT method (where CT is the cycle threshold). We defined >1.6 copies as a duplication of the gene.

Evaluation of the Number of *Pvmdr-1* Copies

We measured number of *Pvmdr-1* (*Pfmdr-1*) copies following the same procedure, relative to the single copy of the *β-tubulin*. Briefly, PCRs were conducted in 25-mL volumes in a 96-well plate containing 1X HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne), 0.3 mM of each forward and reverse primer (*Pvmdr-1*, 5′-GCAACTCCATAAAGAACAACATCA-3′ and 5′-TTTGAGAAGAAAAACCATCTTCG-3′; *β-tubulin*, 5′-CATGTTCGTTAAGATTTCCTGGT-3′ and 5′-GTTAGTGGTGCAAAACCAATCA-3′), and 4 mL of template DNA. Amplifications were performed under the following conditions: 94°C for 15 min, followed by 45 cycles of 94°C for 15 s, 59°C for 30 s, and 72°C for 30 s. The number of *Pvmdr-1* copies of each sample was measured in triplicate relative to a standard curve by using 6 standards of mixed plasmids cloned into TOPO cloning as described for *Pfmdr*-1 (from the standard-1, 1:1 ratio of *Pvmdr-1* and *β-tubulin* to the standard-6, 6:1 ratio of *Pvmdr-1* and *β-tubulin*) by the ΔCT method (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/20/10/14-0411-Techapp1.pdf). We defined >1.6 copies as a duplication of the gene.

Statistical Analysis

Microsoft Excel 2010 (Microsoft, Redmond, WA, USA) and MedCalc software (v9.1.0.1, Mariakerke, Belgium) were used for data analysis. Categorical variables were compared by χ^2 test, and continuous variables were compared by using the 1-way analysis of variance or Mann-Whitney U test. We considered p values <0.05 as significant.

Ethical Approval

We obtained ethics clearance for the samples used in this study from National Ethics Committee in Cambodia (Ministry of Health), in Madagascar (Ministry of Health), in Sudan (Ministry of Health), and in France (National Reference Center for Malaria). All patients or their parents/guardians provided informed written consent.

Results

Global Distribution of the Number of *Pvmdr-1* Copies

We collected and analyzed 607 *P. vivax* isolates from areas to which the parasite was endemic from a total of 492 residents (117 in South America;, 117 in Asia; and 258 in Africa) and 115 travelers from France to South America (41 travelers), Asia (60), or Africa (14). The number of *Pvmdr-1* copies ranged from 1 to 5 copies (mean 1.28, 95% CI 1.22–1.34; median 1.05 [interquartile range (IQR) 0.84–1.53]) and was distributed as follows: 75% isolates had 1 copy, 18% had 2 copies, 6% had 3 copies, 1.6% had 4 copies, and 0.4% had 5 copies. The frequency of *Pvmdr-1*–amplified isolates was significantly higher in samples from South America (83 [53%] of 158) than in samples from Asia (60 [34%] of 177, $p = 10^{-3}$) or Africa (11 [4.0%] of 272, $p<10^{-5}$). The mean number of *Pvmdr-1* copies was also higher in isolates from South America (1.8) than in isolates from Asia (1.3, p = 0.0007) or Africa (0.9, $p<10^{-5}$). Number of copies differed significantly between *P. vivax* isolates from residents and those from travelers. In South America, the proportion of isolates with >1 copy of *Pvmdr-1* was significantly lower in travelers (34% vs. 59%, odds ratio [OR] 0.4 [95% CI 0.2–0.8], p = 0.007); in Africa, this proportion was significantly higher in travelers (57% vs. 1%, OR 113, 95% CI 24–536, p<0.0001) (Tables 1, 2; Figure 1).

Amplification of *Pvmdr-1* in Isolates from Residents

Number of copies in isolates from residents differed significantly among continents (Table 3). The mean

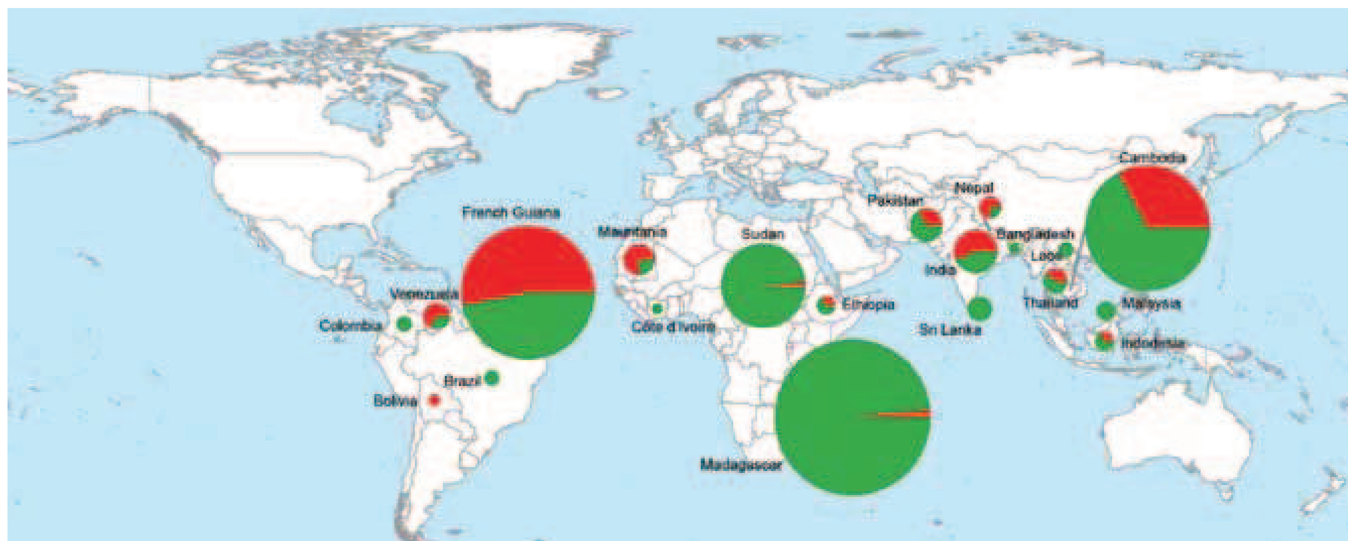

Figure 1. Geographic distribution of *Plasmodium vivax* isolates with 1 multidrug resistance-1 (*mdr-1)* copy (green) and isolates with >1 *mdr-1* copies (red) in 607 samples collected in South America, Asia, and Africa during 1997–2010.

Table 1. Variation in number of copies of *Plasmodium vivax mdr-1* gene in 607 isolates from residents of and travelers from France to malaria-endemic areas, South America, Asia, and Africa, 1997–2009

Variable	Residents	Travelers	Total	p value
No. isolates	492	115	607	–
Origin of isolate, no. (%)				
South America	117 (24)	41 (36)	158 (26)	–
Asia	117 (24)	60 (52)	177 (29)	
Africa	258 (52)	14 (12)	272 (45)	
Isolates with >1 *Pvmdr-1* copy				
No. (%; 95% CI)	109 (22; 18–26)	45 (39; 30–49)	154 (25; 22–29)	$<10^{-3}$*
Range	1–5	1–3	1–5	
Copies of *Pvmdr-1*, no. (%)				
1	383 (77.9)	70 (61)	453 (75)	$<10^{-8}$†
2	63 (12.8)	44 (38)	108 (18)	
3	34 (6.9)	1 (1)	35 (6)	
4	10 (2.0)	0	10 (1.6)	
5	2 (0.4)	0	2 (0.4)	
Mean (95% CI)	1.27 (1.21–1.34)	1.32 (1.22–1.41)	1.28 (1.22–1.34)	0.57‡

*χ^2 test.
†1-way analysis of variance.
‡Mann-Whitney U test.

number of *Pvmdr-1* copies was highest in isolates from South America (2.04, p$<10^{-5}$ vs. Asia and Africa), followed by Asia (1.32, p$<10^{-4}$ vs. Africa) and Africa (0.90 for Madagascar and 1.0 for Sudan). In South America, the proportion of isolates with >1 *Pvmdr-1* copies was also more frequent (59%) than in Asia (33%, OR 0.33, 95% CI 0.2–0.6, p = 10^{-4}) and Africa (1%, OR 0.008, 95% CI 0.002–0.02, p$<10^{-5}$ vs. South America; OR = 0.02, 95% CI 0.007–0.08, p$<10^{-4}$ vs. Asia).

Amplification of *Pvmdr-1* in Isolates from Travelers

The mean number of *Pvmdr-1* copies was similar for travelers returning to France from South America or Asia and did not change by year of collection: 1997–2000 (1.42 and 1.24, respectively), 2001–2005 (1.17 and 1.38), and 2006–2009 (1.20 and 1.49). In contrast, for travelers returning to France from Africa, the mean number of *Pvmdr-1* copies increased in samples collected more recently: 0.60 during 1997–2000, 1.02 during 2001–2005,

Table 2. Variation in number of copies of *Plasmodium vivax mdr-1* gene among isolates from residents of malaria-endemic continents and from travelers from France to those areas, 1997–2010

Continent	Residents	Travelers	Total	p value
South America				
Isolates with >1 *Pvmdr-1* copy	117	41	158	
No. (%; 95% CI)	69 (59; 50–68)	14 (34; 20–51)	83 (53; 44–60)	0.01*
Range	1–5	1–2	1–5	
Copies, no. (%)				
1	48 (41)	27 (66)	75 (47)	10^{-4}*
2	26 (22)	14 (34)	40 (25)	
3	32 (27)	0	32 (20)	
4	9 (8)	0	9 (6)	
5	2 (2)	0	2 (1)	
Mean (95% CI)	2.0 (1.8–2.2)	1.2 (1.0–1.3)	1.8 (1.7–2.0)	$<10^{-5}$†
Asia				
Isolates with >1 *Pvmdr-1* copy	117	60	177	
No. (%; 95% CI)	37 (32; 23–45)	23 (38; 24–57)	60 (34; 26–44)	0.5*
Range	1–4	1–3	1–4	
Copies, no. (%)				
1	80 (68)	37 (62)	117 (66)	0.68*
2	34 (29)	22 (37)	56 (32)	
3	2 (2)	1 (2)	3 (2)	
4	1 (1)	0	1 (1)	
Mean (95% CI)	1.3 (1.2–1.4)	1.4 (1.3–1.5)	1.3 (1.2–1.4)	0.41†
Africa				
Isolates with >1 *Pvmdr-1* copy	258	14	272	
No. (%; 95% CI)	3 (1; 0.2–3)	8 (57; 24–100)	11 (4; 2–7)	$<10^{-8}$*
Range	1–2	1–2	1–2	
Copies, no. (%)				
1	255 (99)	6 (43)	261 (96)	$<10^{-8}$*
2	3 (1)	8 (57)	11 (4)	
Mean (95% CI)	0.9 (0.9–1.0)	1.3 (1.1–1.6)	0.9 (0.9–1.0)	$<10^{-8}$†

*χ^2 test.
†1-way analysis of variance.

Table 3. Variation in number of copies of *Plasmodium vivax mdr-1* gene in isolates from residents of selected Asian and African countries

Variable	Country, years of sample collection				Total	p value
	French Guiana, 2000–2003	Cambodia, 2010	Madagascar, 2006–2007	Sudan, 2007		
No. isolates	117	117	199	59	492	
Isolates with >1 *Pvmdr-1* copy						
No. (%; 95% CI)	69 (59; 46–75)	37 (32; 23–45)	2 (1; 0.1–3.6)	1 (2; 0.4–9.5)	109 (22; 18–26)	$<10^{-10}$*
Range	1–5	1–4	1–2	1–2	1–5	
Copies, no. (%)						$<10^{-10}$*
1	48 (41)	80 (67)	198 (99)	57 (98)	383 (77.9)	
2	26 (22)	34 (30)	1 (1)	2 (2)	63 (12.8)	
3	32 (27)	2 (2)	0	0	34 (6.9)	
4	9 (7)	1 (1)	0	0	10 (2.0)	
5	2 (3)	0	0	0	2 (0.4)	
Mean (95% CI)	2.04 (1.85–2.24)	1.32 (1.22–1.43)	0.90 (0.84–0.91)	1.00 (0.94–1.05)	1.27 (1.21–1.34)	$<10^{-10}$†

*χ^2 test.
†1-way analysis of variance.

and 1.48 during 2006–2009 (OR 0.15, 95% CI 0.03–0.71, p = 0.049). The proportion of isolates with >1 copies of *Pvmdr-1* did not differ significantly among continents over time (Table 4).

Amplification of *Pvmdr-1* and *Pfmdr-1* in Isolates from Cambodia and Malagasy

We compared the distribution profile of the number of *mdr-1* copies of *P. falciparum* and *P. vivax* isolates in 2 different settings. In both Cambodia and Madagascar, the number of *mdr-1* copies did not differ between the 2 species (Figure 2). In Cambodia, where mefloquine has been widely used for >25 years, the mean of number of *mdr-1* copies was 1.34 for *P. falciparum* (n = 88, 95% CI 1.22–1.47, range 1–3) and 1.34 for *P. vivax* (n = 129, 95% CI 1.24–1.44, range 1–4, p = 0.52). In contrast, in Madagascar where mefloquine has never been recommended and has been barely used, the mean number of *mdr-1* copies was 0.92 for *P. falciparum* (n = 350, 95% CI 0.90–0.94, range 1–2) and 0.90 for *P. vivax* (n = 201, 95% CI 0.87–0.93, range 1–2, p = 0.21).

Discussion

Developed in the 1970s at the US Department of Defense's Walter Reed Army Institute of Research as a synthetic analog of quinine (*25*), mefloquine was introduced in 1983 in Thailand to replace chloroquine as first-line treatment for falciparum malaria (*26*). Since then, mefloquine alone or in combination with artesunate has been widely used, especially in Southeast Asia (including Cambodia) and South America (including French Guiana), where it was introduced for second-line treatment and for chemoprophylaxis in 1990 (*27*). In contrast, mefloquine has not been used extensively in Africa and has not been introduced in Madagascar. Mefloquine has been available for malaria chemoprophylaxis since 1985 in Europe and since 1990 in the United States and has been used by >35 million travelers from France for this indication (*28*,*29*).

Pfmdr-1 gene amplification has been described as the major mechanism of *P. falciparum* mefloquine resistance associated with treatment failure or in vitro resistance (*13–16*). Previous studies, including ours, confirm that *mdr-1* amplification does occur in *P. vivax (17–22)*. In addition, the epidemiologic data in our current study show that in regions where mefloquine has never been used, such as in Madagascar and Sudan, amplification in *Pvmdr*-1 is rare (1% and 2% of total isolates, respectively), whereas in areas with current or past intense use of mefloquine, such as in French Guiana and Cambodia, *Pvmdr*-1 amplification is frequent and detected in 59% and 33% of isolates, respectively, and with a mean of 2 and 1.3 copies, respectively.

Table 4. Variation in number of copies of *Plasmodium vivax mdr-1* gene among isolates from persons from France who reported having traveled during the previous month to malaria-endemic continents, 1997–2010

Variable	South America	Asia	Africa	Total	p value
No. isolates	41	60	14	115	
Isolates with >1 *Pvmdr-1* copy					
No. (%; 95% CI)	14 (34; 18–57)	23 (38; 24–57)	8 (57; 24–100)	45 (39; 30–49)	0.30*
Range	1–2	1–3	1–2	1–3	
Copies, no. (%)					0.49*
1	26 (63)	37 (62)	6 (43)	70 (61)	
2	14 (34)	22 (37)	8 (57)	44 (38)	
3	0	1 (2)	0	1 (1)	
Mean (95% CI)	1.20 (1.05–1.35)	1.40 (1.27–1.53)	1.35 (1.10–1.59)	1.32 (1.22–1.41)	0.12†

*χ^2 test.
†1-way analysis of variance.

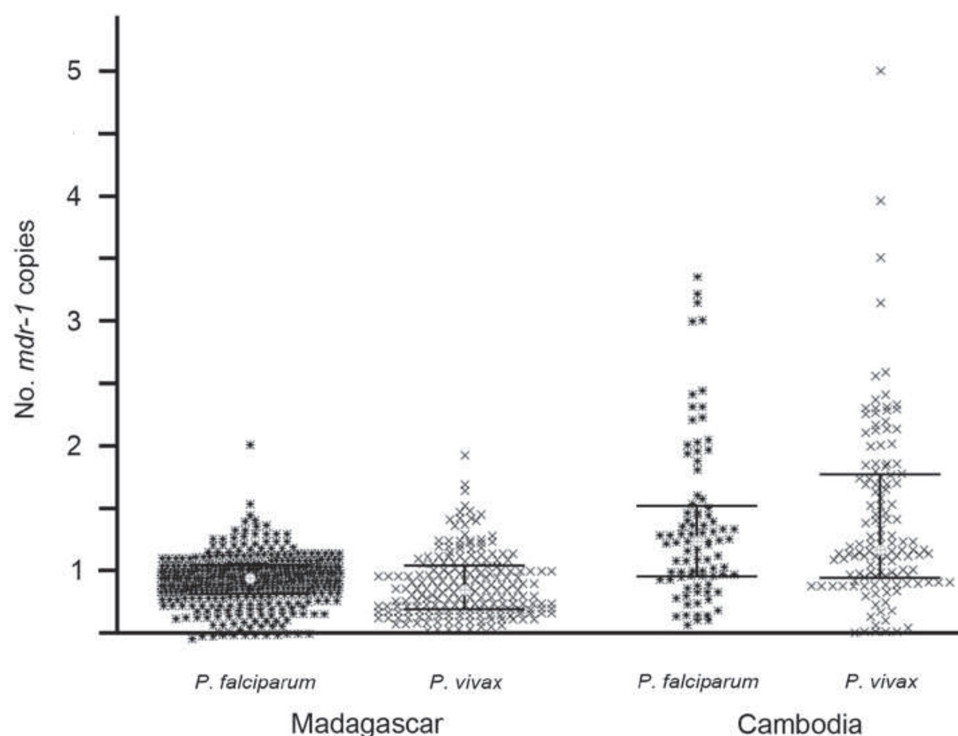

Figure 2. Comparative distribution of numbers of P*lasmodium falciparum* and *P. vivax* mdr-1 copies in isolates collected from residents of Madagascar and Cambodia. Gray dot, median; dark bars, interquartile range (25%–75%).

Both the number of copies and prevalence of *Pvmdr*-1 of isolates with multiple *mdr*-1 copies we observed here are much higher than reported in other studies. For instance, Imwong et al. reported *Pvmdr-1* amplification in 6/66, 2/50, and 1/49 isolates from Thailand, Laos, and Myanmar, respectively (*17*); Jovel et al. observed *Pvmdr-1* amplification in 1/37 in Honduras (*20*); and Lin et al. recently reported 39% and only 4% prevalence in *P. vivax* isolates from Thailand and Cambodia, respectively (*18*). A reason for the discrepancy between observations from Lin et al. in Cambodia and our observations could be because their isolates were collected during 2006–2007 and our samples were collected in 2010, indicating an increase in *Pvmdr-1* amplification over 3 years. Another reason is the location of collection. Indeed, drug resistance and drug pressure markedly differ across Cambodia. Lin et al. studied isolates from southern Cambodia (Kampot Province), whereas we studied isolates from areas in which drugs were highly resistant in western (Pailin Province) and southeastern Cambodia (Kratie Province), where multidrug resistance of *P falciparum* is emerging (*30*) and drug pressure (including artesunate–mefloquine combination) has been intense in recent years. To our knowledge, the previous maximum number of *Pvmdr-1* copies detected was 3 (*18*,*19*); in this study. however, we observed up to 5 copies in isolates from South America and 4 copies in isolates from Southeast Asia. This difference is likely to be due to our real-time PCR approach using 6 standards of mixed plasmids, which enabled detection of *P. vivax* isolates with *mdr-1* amplification with up to 6 copies. This observation also could indicate an ongoing selection of *mdr-1*–amplified parasites. Although, our data need to be confirmed and supported by in vivo data from mefloquine-treated patients or in vitro experiments showing a direct relationship between mefloquine pressure and *P. vivax mdr-1* amplification, our findings advocate for an integrated drug policy whereby all sympatric malaria species are considered regarding treatment efficacy but also drug pressure and selection of resistance.

We were able to assess the number of *Pvmdr-1* copies in isolates collected from travelers returning to France. These data must be analyzed with caution because information about the location of infection might be erroneous, particularly given the fact that relapses from hypnozoites can occur several months after primary infection. We cannot exclude that a patient declaring having returned from Africa was previously infected during a trip to a different location because we did not include this information in the questionnaire administered at recruitment. Nevertheless, we found significant differences between travelers from France and residents from a given geographic origin, especially in isolates from Africa, where most (99%) isolates from residents displayed no amplification, whereas most (57%) isolates from travelers from France had *Pvmdr-1* amplification. We assume this indicates within-host selection by mefloquine prophylaxis, which has been and continues to be widely used among travelers from France who go to malaria-endemic countries. Such pressure does not exist in residents, who usually do not take any prophylaxis.

These data are of concern because they suggest that selection of *Pvmdr-1* amplification is a more rapid process than previously thought, reminiscent to atovaquone resistance. Although selection in travelers from France returning to nonendemic areas bears no transmission risk, chemoprophylaxis and intermittent preventive treatments in malaria-endemic areas might contribute to the emergence of resistant parasites. This possibility certainly warrants further investigation.

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Ms Khim is a PhD student at the Malaria Molecular Epidemiology Unit in Institut Pasteur in Cambodia. Her research interests include clinical epidemiology and antimalarial drug resistance in vivax malaria and more generally are focused on developing molecular tools for improving the surveillance of resistance to antimalarial drugs in Cambodia.

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OBJECTIF 2: de mettre en place des outils biologique et moléculaires, permettant de mieux définir l'épidémiologie des parasites résistants, en particulier la quinine et les dérivés de l'artémisinine

O2.1. Etude de l'association entre le polymorphisme du gène candidat *Plasmodium falciparum Na+/H+ exchanger* (*pfnhe-1*) et la susceptibilité *in vitro* de *P. falciparum* à la quinine

Le paludisme est une maladie provoquée par des hématozoaires appartenant au genre *Plasmodium*. Deux espèces parmi les cinq qui infectent l'homme, *Plasmodium falciparum* et *Plasmodium vivax*, ont un impact majeur en santé publique. *P. falciparum* provoque une infection aiguë, potentiellement mortelle chez les sujets peu ou non immuns. *P. vivax,* longtemps considéré comme provoquant des infections bénignes, est maintenant reconnu comme un pathogène susceptible de provoquer des complications graves, responsables de décès (Anstey et al. 2009). En cas d'infection palustre, un antipaludique efficace doit être administré le plus rapidement possible pour éviter les complications graves. Le traitement précoce des patients a comme conséquence supplémentaire de réduire la probabilité de transmission du parasite au moustique. En l'absence de vaccin, les antipaludiques jouent un rôle central dans la lutte contre le paludisme. Parmi eux, la quinine occupe une place de choix. Premier antipaludique utilisé, depuis plus de 200 ans, la quinine est bien souvent réservée aux traitements des cas sévères de paludisme à *P. falciparum*. Malheureusement, comme c'est le cas pour de nombreuses infections microbiennes, la pression médicamenteuse exercée au cours des dernières décennies a sélectionné des parasites résistants.

Dans ce contexte, la surveillance de l'efficacité des antipaludiques est primordiale. Bien souvent, la mise en évidence d'association entre le polymorphisme au sein de gènes supposés être impliqués dans la résistance et un phénotype clinique (échecs thérapeutique, délai de clairance) ou un phénotype biologique (diminution de la sensibilité *in vitro* d'isolats) constitue une étape cruciale pour améliorer la surveillance de l'émergence et de la diffusion des souches résistantes de *Plasmodium falciparum*. Bien que nous ayons connu un certain succès dans l'identification des marqueurs moléculaires prédictifs de la résistance à plusieurs antipaludiques (*Pfcrt, Pfmdr-1, Pfdhfr, Pfdhps*), pour un certain nombre de gènes candidats, les données disponibles restent conflictuelles. C'est le cas du gène *pfnhe-1* (*P. falciparum Na+/H+* exchanger-1) situé sur le chromosome 13 qui semble associé à la sensibilité réduite à la quinine. Plusieurs études ont montré une association significative entre le polymorphisme du microsatellite ms4760 associé au gène *pfnhe-1* et la réponse *in vitro* des parasites à la quinine, alors que d'autres observations contradictoires ont démontré une absence d'association, probablement liées à l'origine géographique des isolats étudiés.

Nous avons, dans les travaux présentés ci-dessous, étudié sur un nombre large d'isolats cette association entre polymorphisme génomique et susceptibilité *in vitro*. Ce travail s'est fait en collaboration avec l'Institut Pasteur du de Madagascar et le Centre National de Reference du Paludisme à Paris.
Dans cette première étude (**article 3**), nous avons séquencé le gène candidat *Plasmodium falciparum* Na^+/H^+ exchanger (*pfnhe-1*; PF13_0019) de 595 parasites de *Plasmodium falciparum* circulant dans les régions endémiques de l'océan Indien (Madagascar et Comores). Nous avons observé plus de nombre de répétitions du motif DNNND (block II) parmi les isolats des Comores, montrant une association significante avec la susceptibilité *in vitro* des isolats à la quinine.

Article 3: Andriantsoanirina, V., **N. Khim**, A. Ratsimbasoa, B. Witkowski, C. Benedet, L. Canier, C. Bouchier, M. Tichit, R. Durand and D. Menard (2013). *Plasmodium falciparum Na+/H+ exchanger* (*pfnhe-1*) genetic polymorphism in Indian Ocean malaria-endemic areas." Am J Trop Med Hyg 88: 37-42).

Dans la deuxième étude (article 4), nous avons intégré 1508 séquences de *ms4760* du gène *pfnhe-1* disponibles dans le GenBank y compris les 595 séquences de la première étude pour effectuer une analyse globale du poylmorphisme des allèles *ms4760* du gène *pfnhe-1* comme un marqueur moléculaire candidat de la résistance à la quinine. Nous avons trouvé que la prévalence des profils *ms4760* du gène *pfnhe-1* était significativement différente entre les isolats venant de différents continents. Nous avons conclu que le polymorphisme *ms4760* du gène *pfnhe-1* ne pouvaient pas être proposés comme le marqueur moléculaire pour la résistance à la quinine.

Article 4: Menard, D., V. Andriantsoanirina, **N. Khim**, A. Ratsimbasoa, B. Witkowski, C. Benedet, L. Canier, O. Mercereau-Puijalon and R. Durand (2013). Global analysis of *Plasmodium falciparum Na(+)/H(+) exchanger* (*pfnhe-1*) allele polymorphism and its usefulness as a marker of *in vitro* resistance to quinine." Int J Parasitol Drugs Drug Resist 3: 8-19.

Commentaires

La méthodologie utilisée a été la même que celle développée précédemment, nous nous sommes intéressés au gène *pfnhe-1* de *Plasmodium falciparum* qui est le gène candidat d'association avec la résistance à la quinine.

Dans la première étude menée à Madagascar (Andriantsoanirina et al. 2013), 595 isolats provenaient des Comores et avaient été collectés sur papiers buvards en 2006 (N=250), zones de forte résistance à la chloroquine et à la pyriméthamine-sulfadoxine et à Madagascar chez les sujets symptomatiques entre 2006 et 2007 (N=345), région où la résistance aux antipaludiques est faible. Les produits amplifiés ms4760 *de pfnhe-1* ont été séquencés par la méthode Sanger. Les séquences obtenues ont été alignées avec la séquence de référence *P. falciparum* 3D7 (sodium/hydrogen exchanger, Na+, H+ antiporter, PF13_0019, XM_001349726).

Dans l'étude analysée globale (Menard et al. 2013), 1508 isolats ont été analysés pour le gène *ms4760*. Une partie de ces isolats provenanient des parasites cultivés et adaptés ou des souches de référence provenant de plusieures régions géographiques récupérés à partir de GenBank (dernière mise à jour le 15 juin 2012) ou de publications.

Résultats

Les données présentées dans l'article 3 *(Andriantsoanirina et al. 2013)* font suite à une précédente étude conduite en 2010 *(Andriantsoanirina et al. 2010)*. Nous avons constaté que les parasites venant des Comores présentaient plus de répétition DNNND (block II) par rapport aux isolats venant de Madagascar. Ce nombre variait de zéro à quatre, tandis que le nombre de répétition NHNDNHNNDDD variait de zéro à trois. Le ratio moyen DNNND/NHNDNHNNDDD était significativement plus élevé aux Comores qu'à Madagascar ($P<0.001$). La prévalence des profils *ms4760* des isolats en fonction de la localisation géographique dans les deux pays était également significativement différente.

Les analyses bio-statistiques effectuées dans la seconde étude *(Menard et al. 2013)* montrait une grande diversité génétique globale de *ms4760*. Les isolats africains présentaient un grand nombre des allèles différents, suivi par l'Asie et l'Amérique du Sud. Par ailleurs, quelques allèles étaient partagés entre les 3 continents, les autres plus restreint à l'Asie et/ou à l'Afrique. La répartition géographique et la prévalence des profils *ms4760*, définit par les variations du block II et du block V, différait aussi significativement entre les continents. Il est important à noter que le rapport moyen DNNND/DDNHNDNHNND qui a été proposé par certains auteurs comme associé à la résistance à la quinine, était significativement plus élevé en Asie qu'en Afrique. La prévalence des profils *ms4760* en fonction de localisation géographique des isolats (les continents et les pays) était significativement différente entre les continents ($p<0.0001$).

Ces données montrent que les populations asiatiques et africaines sont clairement différentes. La limite de ces études est que la susceptibilité *in vitro* des isolats à la quinine et la détermination du polymorphisme de *ms4760* ont été effectuée à partir d'isolats adaptés en culture ou de souches de référence, pouvant entrainer un biais par sélection de mutations au cours de la culture en laboratoire. En effet, l'augmentation du nombre de répétions DNNND a été associé avec la résistance *in vitro* à la quinine dans 6 études *(Ferdig et al. 2004, Henry et al. 2009, Meng et al. 2010, Okombo et al. 2010, Pelleau et al. 2011, Sinou et al. 2011)*, non confirmé par d'autres *(Bertaux et al. 2011, Poyomtip et al. 2012, Pradines B. et al. 2010b)*.

Conclusion

La recherche d'association entre le polymorphismes de ms4760 et la résistance à quinine ne semble pas être confirmée *(Andriantsoanirina et al. 2013)* et ne permet pas de proposer une simple méthode de typage moléculaire. Le niveau de la diversité génétique observé dans cette étude a été comparé avec celui découvert

dans les pays africains et non comparable avec celui trouvé dans les pays asiatiques. Le nombre moyen de motif répété DNNND était plus important parmi les isolats venant des Comores par rapport à ceux de Madagascar.

Dans le second article *(Menard et al. 2013)*, l'association entre les différents allèles du gène *pfnhe-1* et la résistance à la quinine a montré des disparités géographiques remarquables. La validité et la fiabilité des *polymorphismes* du gène candidat *pfnhe-1* comme le marqueur moléculaire à la résistance à la quinine semblent restreint aux zones endémiques d'Asie du Sud ou éventuellement des pays d'Afrique de l'Est, suggérant l'importance de l'origine de populations parasitaires. Des études *supplémentaires* seront nécessaires pour mieux définir les mécanismes sous-jacents de la résistance à la quinine.

Article 3

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Short Report: *Plasmodium falciparum* Na^+/H^+ Exchanger (*pfnhe-1*) Genetic Polymorphism in Indian Ocean Malaria-Endemic Areas

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Abstract. To date, 11 studies conducted in different countries to test the association between *Plasmodium falciparum* Na^+/H^+ exchanger gene (*pfnhe-1*; PF13_0019) polymorphisms and *in vitro* susceptibility to quinine have generated conflicting data. In this context and to extend our knowledge of the genetic polymorphism of *Pfnhe* gene, we have sequenced the *ms4760* locus from 595 isolates collected in the Comoros ($N = 250$; an area with a high prevalence of chloroquine and sulfadoxine-pyrimethamine resistance) and Madagascar ($N = 345$; a low drug-resistance area). Among them, 29 different alleles were observed, including 8 (27%) alleles not previously described. Isolates from the Comoros showed more repeats in block II (DNNND), which some studies have found to be positively associated with *in vitro* resistance to quinine, compared with isolates from Madagascar. Additional studies are required to better define the mechanisms underlying quinine resistance, which involve multiple gene interactions.

Quinine (QN), a natural quinoline derivative compound found in *Cinchona* bark, has been used for centuries in malaria-endemic regions.[1] To date, resistance to QN remains particularly patchy and rare,[2–12] and only few cases of clinical failure have been reported in Asia and South America. The mechanism underlying QN resistance is not well-understood, and it is probably complex and multigenic. Since the seminal work by Ferdig and others[13] in 2004, 11 studies have been conducted in different countries to evaluate implication of *ms4760* polymorphisms in QN resistance,[14–24] and conflicting data have been reported, likely because of the different geographical origin of parasites (implying different genetic backgrounds), the type of parasites used (fresh isolates, culture-adapted strains, and reference lines), and the method used to assess *in vitro* QN susceptibility.[21,25]

In this context and to extend our previous work regarding *ms4760* polymorphisms in *Plasmodium falciparum* parasites circulating in malaria-endemic areas in the Indian Ocean,[14] we have analyzed *ms4760* sequences from 595 isolates (Madagascar, $N = 345$; Comoros, $N = 250$).

P. falciparum isolates from Madagascar were collected in 2006 and 2007 as part of the surveillance of antimalarial drug resistance from symptomatic malaria-infected patients before treatment in 14 health centers (northwest: Antohihy, Analalava, Mahajanga, and Maevatanana; central west: Tsiroanomandidy, Miandrivazo, and Morondava; southwest: Ihosy, Ejeda, and Toliara; northeast: Andapa; central east: Toamasina and Moramanga; southeast: Faranfagana). Comorian isolates were collected from finger prick onto filter paper in 2006 in six different sites (Grande Comore: Moroni and Foumbouni; Mohéli: Fomboni and Wanani; Anjouan: Pomoni and Domoni) (Figure 1). Informed written consent was provided by all patients or their parents/guardians before inclusion in the study, and blood collections were conducted in accordance to the Ethics Committee of the Ministries of Health of Madagascar and the Comoros (N°007/SANPF/2007; registration number ISRCTN36517335).

Parasite DNA was extracted from blood spots with Instagene matrix (Bio-Rad, Marnes la Coquette, France) according to the manufacturer's instructions or directly from 100 μL infected blood by the phenol-chloroform method.[26] The parasite species was confirmed by real-time polymerase chain reaction (PCR) as described in the work by Mangold and others.[27] Amplification and sequencing of the *ms4760* locus in the *P. falciparum* Na^+/H^+ exchanger gene (pfnhe-1) was performed in accordance with the protocol described earlier.[14] *pfnhe-1 ms4760* alleles were constructed from a full sequence presenting an unambiguous single-allele signal at all positions and used *P. falciparum* 3D7 (sodium/hydrogen exchanger, Na^+, H^+ antiporter, PF13_0019, XM_001349726) as the reference.

Genetic diversity was assessed by Nei's unbiased expected heterozygosity (H_e) from haploid data and calculated as $H_e = [n/(n-1)][1 - p_i]$ (n = the number of isolates sampled; p_i = the frequency of the ith allele).[28] Population genetic differentiation was measured using Wright's F statistics (F_{st}).[29] A P value < 0.05 was considered statistically significant.

Nucleotide sequences of new *ms4760* haplotypes were deposited in the GenBank database under accession numbers from JX472441 to JX472448.

Among the 595 *P. falciparum* isolates (Madagascar, $N = 345$; Comoros, $N = 250$), 29 different alleles were observed, including 8 alleles not previously described (*ms4760*-90 to *ms4760*-97) (Table 1). *ms4760*-1 was the most prevalent (180/595; 30.3%) followed by *ms4760*-3 (96/595; 16.1%), *ms4760*-7 (77/595; 12.9%), and *ms4760*-6 or *ms4760*-9 (43/595; 7.2%); 15 *ms4760* alleles (*ms4760*-1, *ms4760*-2, *ms4760*-3, *ms4760*-6, *ms4760*-7, *ms4760*-8, *ms4760*-9, *ms4760*-12, *ms4760*-27, *ms4760*-29, *ms4760*-30, *ms4760*-35, *ms4760*-91, *ms4760*-95, and *ms4760*-96) were distributed in both countries, whereas others were exclusively found in Madagascar ($N = 10$; *ms4760*-19, *ms4760*-22, *ms4760*-31, *ms4760*-32, *ms4760*-33, *ms4760*-42, *ms4760*-92, *ms4760*-93, *ms4760*-94, and *ms4760*-97) or the Comoros ($N = 4$; *ms4760*-5, *ms4760*-14, *ms4760*-34, and *ms4760*-90). Details are given in Table 1, and multiple amino acid sequence alignments are shown in Figure 2.

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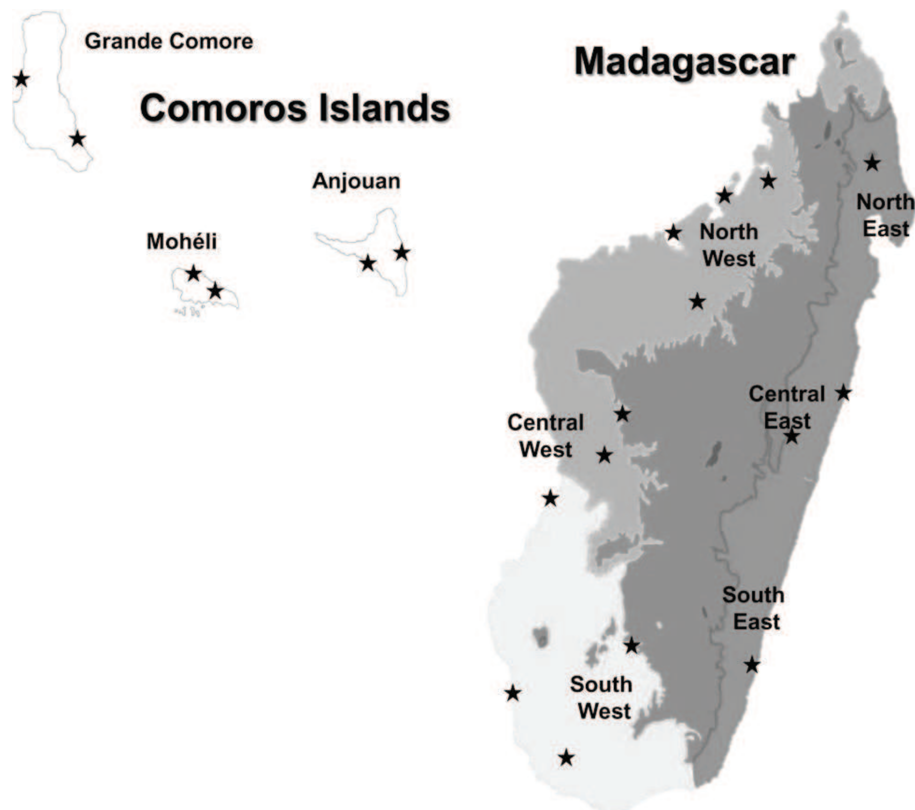

FIGURE 1. Samples collection sites in the Comoros Islands and Madagascar from 2006 to 2007.

The distribution and prevalence of the alleles were significantly different between countries ($P < 0.0001$) and within country only for isolates from the Comoros (between Anjouan/Mohéli and Grande Comore, $P = 0.003$ and $P = 0.02$, respectively).

According to the number of repeats in block II (DNNND) and block V (NHNDNHNNDDD), which have been associated with modulation of *in vitro* susceptibility to QN,[13] *ms4760* alleles were grouped in 12 different profiles (from *ms4760*-A to *ms4760*-L) as presented in Table 2. The number of repeats in block II (DNNND) varied from zero (*ms-4760*-A) to four (*ms4760*-K and *ms4760*-L), whereas the number of repeats in block V (NHNDNHNNDDD) varied from zero (*ms4760*-E) to three (*ms4760*-D and *ms4760*-H); 73% of isolates were grouped in three profiles: *ms-4760*-C ($[DNNND]_1$; $[NHNDNHNNDDD]_2$; 20.5%), *ms-4760*-G ($[DNNND]_2$; $[NHNDNHNNDDD]_2$; 32.3%), and *ms-4760*-I ($[DNNND]_3$; $[NHNDNHNNDDD]_1$; 20.3%). Seven profiles (*ms-4760*-B, -C, -D, -F, -G, -I, and -J) were found in both countries, four profiles (*ms-4760*-A, -E, -H, and -L) were found only in Madagascar, and one profile (*ms-4760*-H) was found only in the Comoros. The mean number of DNNND repeats was significantly higher in the Comoros (2.21) compared with Madagascar (1.93; $P < 0.001$). Inversely, the number of NHNDNHNNDDD repeats was significantly lower in the Comoros (1.60) compared with Madagascar (1.73; $P = 0.005$). Consequently, the mean ratio of DNNND/NHNDNHNNDDD repeats was significantly higher in the Comoros (1.77 versus 1.47; $P < 0.001$). The prevalence of the *pfnhe-1 ms4760* profiles according to the geographical location of the isolates (country and region) significantly differed between the two countries ($P < 0.0001$). In both countries, three profiles were predominant (*ms-4760*-G, 32.3%; *ms-4760*-I, 20.3%; *ms-4760*-C, 20.5%). Two profiles (*ms-4760*-I, $P < 0.0001$; *ms-4760*-K, $P = 0.006$) were significantly more frequent in the Comoros, and one profile (*ms-4760*-C, $P = 0.02$) was significantly more frequent in Madagascar.

Genetic diversity, assessed by Nei's unbiased expected heterozygosity (H_e), was similar between countries (Madagascar = 0.84, ranging from 0.75 for the southeast area to 0.85 for the central west area; Comoros = 0.85, ranging from 0.80 for Grande Comore to 0.87 for Mohéli). However, the degree of genetic differentiation of the *ms4760* profiles within parasite populations, estimated by F_{st} values, indicated a large divergence between Grande Comore populations and Malagasy populations from the northwest, central east, west, and southwest areas (Table 3).

The data represented here are an extension of our previous study performed in 2010.[14] By using a large number of *P. falciparum* isolates from Indian Ocean malaria-endemic areas (Comoros and Madagascar), we confirm the extended polymorphisms of *ms4760* allele in *pfnhe-1* gene in this region. Among the 595 studied sequences, we have observed 29 different alleles, including 8 new alleles (27%). By compiling our data with previous published sequences available in GenBank,[14–24] we estimate that 101 different *ms4760* alleles have been described to date. However, in most publications, the numbering of the *ms4760* alleles did not always taking into account the previously described alleles, making data comparison difficult. This finding raises the need to establish a standard nomenclature for *ms4760* alleles.

TABLE 1
Distribution of ms4760 alleles among Indian Ocean isolates collected in 2006–2007

Allele ms4760	No. DNNND repeats	No. NHNDNHNNDDD repeats	No. of isolates: Comoros Islands: Anjouan *n*	%	Grande Comore *n*	%	Mohéli *n*	%	Total *n*	%	Madagascar: Northwest *n*	%	Northeast *n*	%	Central west *n*	%	Central east *n*	%	Southwest *n*	%	Southeast *n*	%	Total *n*	%	Indian Ocean *n*	%	GenBank accession numbers
ms4760-1	2	2	19	28	43	40	17	23	79	31.6	23	41	4	25	44	28	11	25	10	20	9	41	101	29.3	180	30.3	
ms4760-2	1	1	1	1			1	1	2	0.8	3	5	1	6	4	3	4	9	1	2			13	3.8	15	2.5	
ms4760-3	1	2	9	13	7	6	11	15	27	10.8	6	11	5	31	30	19	10	23	14	28	4	18	69	20.0	96	16.1	
ms4760-5	4	1			5	5	1	1	6	2.4															6	1.0	
ms4760-6	2	1	5	7	6	6	5	7	16	6.4	4	7			13	8	2	5	7	14	1	5	27	7.8	43	7.2	
ms4760-7	3	1	8	12	13	12	13	17	34	13.6	6	11	2	13	19	12	8	18	6	12	2	9	43	12.5	77	12.9	
ms4760-8	3	2	1	1					1	0.4					5	3					1	5	6	1.7	7	1.2	
ms4760-9	3	2	4	6	3	3	6	8	13	5.2	6	11	1	6	13	8	1	2	5	10	4	18	30	8.7	43	7.2	
ms4760-12	1	3			8	7	1	1	9	3.6			1	6	5	3	1	2	1	2			8	2.3	17	2.9	
ms4760-14	3	1			2	2			2	0.8															2	0.3	
ms4760-19	2	2													2	1							2	0.6	2	0.3	
ms4760-22	2	3															2	5					2	0.6	2	0.3	
ms4760-27	2	2	2	3	3	3	1	1	6	2.4	3	5							1	2			4	1.2	10	1.7	
ms4760-29	2	1			9	8	3	4	12	4.8					2	1							2	0.6	14	2.4	
ms4760-30	1	2	2	3			1	1	3	1.2	1	2			6	4			2	4			9	2.6	12	2.0	
ms4760-31	1	2													4	3	1	2					5	1.4	5	0.8	
ms4760-32	2	1											1	6	1	1							2	0.6	2	0.3	
ms4760-33	0	2													1	1							1	0.3	1	0.2	
ms4760-34	4	1			1	1			1	0.4															1	0.2	
ms4760-35	1	1					1	1	1	0.4					1	1	1	2					2	0.6	3	0.5	
ms4760-42	1	1																			1	5	1	0.3	1	0.2	
ms4760-90	3	1	3	4	1	1	1	1	5	2.0															5	0.8	JX472441
ms4760-91	1	2			1	1	3	4	4	1.6			1	6	2	1			1	2			4	1.2	8	1.3	JX472442
ms4760-92	1	1													1	1							1	0.3	1	0.2	JX472443
ms4760-93	1	2															1	2					1	0.3	1	0.2	JX472444
ms4760-94	2	0																	2	4			2	0.6	2	0.3	JX472445
ms4760-95	3	1	7	10	4	4	9	12	20	8.0					2	1	1	2					3	0.9	23	3.9	JX472446
ms4760-96	3	1	6	9	2	2	1	1	9	3.6	3	5			1	1	1	2					5	1.4	14	2.4	JX472447
ms4760-97	4	2									1	2			1	1							2	0.6	2	0.3	JX472448
Total				67		108		75		250		56		16		157		44		50		22		345		595	

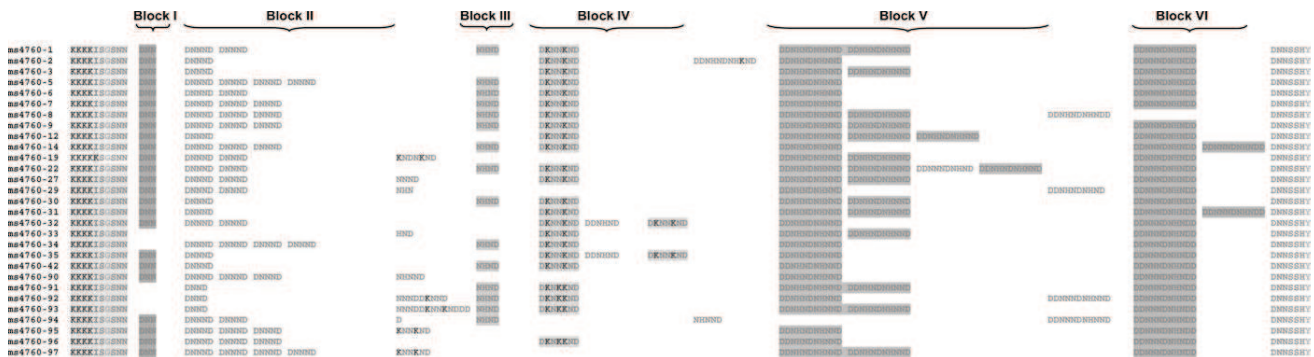

FIGURE 2. Amino acid alignment of 29 *ms4760* haplotypes found in the Comoros Islands and Madagascar from 2006 to 2007.

As expected and found in previous studies, four alleles (*ms4760*-1, *ms4760*-3, *ms4760*-6, and *ms4760*-7) were predominant in both countries.[14,15,17–22] However, significant differences in the distribution and prevalence of allele were observed both between countries and within sites in the Comoros (Table 1). The genetic diversity of the *ms4760* allele observed in our study was similar between both countries (0.84 and 0.85), and it was comparable with the genetic diversity previously described in African (Congo = 0.76, Uganda = 0.79, and Kenya = 0.66)[15,17,20] and Indian isolates (0.68)[23] and significantly higher than the diversity found in Asian isolates (China/Myanmar = 0.68, $P = 0.04$; Vietnam = 0.49, $P < 0.0001$).[19,22] This situation is likely reflecting the level of malaria transmission, but it also could be related to the prevalence of resistant parasites to quinoline antimalarial drugs. This latter hypothesis is strengthened by our data, which show that isolates from the Comoros (an area with a high prevalence of antimalarial drugs resistance, although specific data about QN resistance are lacking) had significantly more repeats in block II (DNNND) than those isolates from Madagascar (a low drug-resistance area); these findings are consistent with some previous findings observed in culture-adapted parasites from Asia,[13,18,19,21,22] India,[23] and East Africa.[20]

In conclusion, current observations from molecular surveys that aimed to define an association between potential contributors to QN resistance, such as *ms4760* allele polymorphism, have generated conflicting data and do not allow for proposing a simple molecular typing methodology of global application based on this molecular marker. The level of genetic diversity observed in the present study was comparable with the level found in African countries and not comparable with the level found in Asian countries, where the *pfnhe-1* polymorphisms seemed more often usable as molecular markers of QN resistance.[19,22] The higher mean number of DNNND repeats found in isolates from the Comoros compared with Madagascar underlined the importance of the geographical origin of parasites, even at this regional level. Additional studies are required to better define the mechanisms underlying QN resistance, which involve multiple gene interactions.

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TABLE 2

Pfhne-1 ms4760 profile groups according to the number of repeats in block II (DNNND) and block V (DDNHNDNHNND) and the geographical location of the isolates

ms4760 alleles number	ms4760 profiles	No. of allele	Block II (DNND)	Block V (DDNHNDNHNND)	Frequency (%)	
					Comoros Islands	Madagascar
33	ms4760-A	1	0	2	0	0.3
2, 35, 42, 92	ms4760-B	4	1	1	1.2	4.9
3, 30, 31, 91, 93	ms4760-C	5	1	2	13.6	25.5
12	ms4760-D	1	1	3	3.6	2.3
94	ms4760-E	1	2	0	0	0.6
6, 29, 32	ms4760-F	3	2	1	11.2	9.0
1, 19, 27	ms4760-G	3	2	2	34.0	31.0
22	ms4760-H	1	2	3	0	0.6
7, 14, 90, 95, 96	ms4760-I	5	3	1	28	14.8
8, 9	ms4760-J	2	3	2	5.6	10.4
5, 34	ms4760-K	2	4	1	2.8	0
97	ms4760-L	1	4	2	0	0.6

Table 3
Pairwise population genetic distances (F_{st} according to Weir and Cockerham)[29]

	Comoros Islands		Madagascar					
	Mohéli	Anjouan	Northeast	Northwest	Central east	Central west	Southeast	Southwest
Comoros Islands								
Grande Comore	0.037	0.003	0.033	0.0014*	0.0014*	0.0014*	0.020	0.0014*
Mohéli		0.346	0.490	0.018	0.114	0.0389	0.232	0.109
Anjouan			0.58	0.119	0.019	0.003	0.125	0.018
Madagascar								
Northeast				0.115	0.843	0.785	0.479	0.540
Northwest					0.051	0.037	0.380	0.061
Central east						0.183	0.193	0.115
Central west							0.680	0.443
Southwest								0.305
Southeast								

* $P > 0.05$.

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Article 4

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Global analysis of *Plasmodium falciparum* Na^+/H^+ exchanger (*pfnhe-1*) allele polymorphism and its usefulness as a marker of *in vitro* resistance to quinine

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ABSTRACT

The aim of this study was to provide a comprehensive analysis of the worldwide genetic polymorphism of ms4760 alleles of the *pfnhe-1* gene and to discuss their usefulness as molecular marker of quinine resistance (QNR). A new numbering of ms4760 allele, classification grouping ms4760 alleles according to the number of DNNND and DDNHNDNHNND repeat motifs in blocks II and V was also proposed.

A total of 1508 ms4760 sequences from isolates, culture-adapted parasites or reference strains from various geographical regions were retrieved from GenBank (last update on 15th June 2012) or from publications and were used for genetic analyses. The association of different alleles of *pfnhe-1* with resistance to quinoline antimalarial drugs showed marked geographic disparities.

The validity and reliability of candidate polymorphisms in *pfnhe-1* gene as molecular markers of QNR appeared restricted to endemic areas from South Asia or possibly East African countries and needs to be confirmed.

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1. Introduction

Quinine (QN), a natural compound found in *Cinchona* bark, has been used for centuries in malaria endemic regions (Baird, 2005). It is currently recommended for treating severe malaria cases, malaria in pregnant women or as second-line therapy in combination with antibiotic for uncomplicated malaria (World Health Organization, 2010a). Though clinical failures have been reported in Asia and South America in the 1960s and later on, although more rarely in Africa, resistance to QN (QNR) remains particularly punctual and rare (Chongsuphajaisiddhi et al., 1983; Pukrittayakamee et al., 1994, 2000; de Vries et al., 2000; McGready et al., 2000, 2005; Rahman et al., 2001; Adam et al., 2005; Adegnika et al., 2005; Achan et al., 2009; World Health Organization, 2010a,b).

QN, a quinoline derivative, is a monoprotic weak base that accumulates within the low pH environment of the parasite digestive vacuole of *Plasmodium falciparum*. QN presumably acts by interference with the detoxification of heme produced during hemoglobin degradation by *P. falciparum* asexual blood stages, leading to toxic degradation by-products (Hawley et al., 1998). However, the mechanism of QNR is not well known. Several reports have documented associations between *in vitro* susceptibility to QN with other structurally related drugs such as amino-4-quinolines (chloroquine, amodiaquine) or aryl-amino-alcohol (mefloquine, halofantrine), suggesting that a common genetic determinant may affect the parasite response to these antimalarials (Simon et al., 1986; Warsame et al., 1991; Basco and Le Bras, 1992; Brasseur et al., 1992). Particularly, QNR has been associated with mutations in the *P. falciparum multidrug resistance 1* gene (*pfmdr-1*) and the *P. falciparum chloroquine resistance transporter* gene (*pfcrt*) (Wongsrichanalai et al., 2002; Valderramos and Fidock, 2006). More recently, other genetic polymorphisms, such as mutations in the *P. falciparum multi-resistance protein 1* gene (*pfmrp-1*) have been suggested (Mu et al., 2003), but not confirmed (Anderson et al., 2005). However, PfMRP knock-out parasite lines displayed increase susceptibly to several antimalarial drugs, including chloroquine, QN and artemisinin derivatives (Raj et al., 2009). The degree of implication or linkage of the three genes in QNR remains uncertain, probably because additional genes are involved. In 2004, by using quantitative trait loci (QTL) analysis on the genetic cross of the HB3 and Dd2 clones, Ferdig et al. (2004) identified genes associated with QN reduced susceptibility (Ferdig et al., 2004), namely *pfmdr-1* on chromosome 5, *pfcrt* on chromosome 7 and *pfnhe-1* (*P. falciparum* Na^+/H^+ exchanger-1) on chromosome 13. To test for an association of QN response with this latter gene, *pfnhe-1* was resequenced from the HB3 and Dd2 parents and the identified coding frame polymorphisms were surveyed in 71 *P. falciparum* culture-adapted isolates and reference lines from South-East Asia, Africa and Central and South America. Sequences of *pfnhe-1* showed multiple and complex variations. Three point polymorphisms at three separate codons (790 gtc/ttc, 894 aat/aaa, 950 ggg/gtg) and microsatellite variations in three different repeat sequences (msR1, ms3580 and ms4760) were observed (Fig. 1). Moreover, there was a significant association between variations in ms4760 and *in vitro* QN response. One of the eight ms4760 profiles, ms4760-1, was relatively frequent in lines with reduced susceptibility to QN (i.e. higher IC_{90}), but it was also present in fully susceptible parasites. More interestingly, the authors reported that presence of more than 2 DNNND repeat motifs in block II was associated with higher *in vitro* IC_{90} for QN compared with presence of only one repeat (Ferdig et al., 2004).

The physiological role of PfNHE-1 is still debated. In all living organisms, the fundamental homeostatic mechanisms are ubiquitous and vital. These physiological processes which regulate cellular

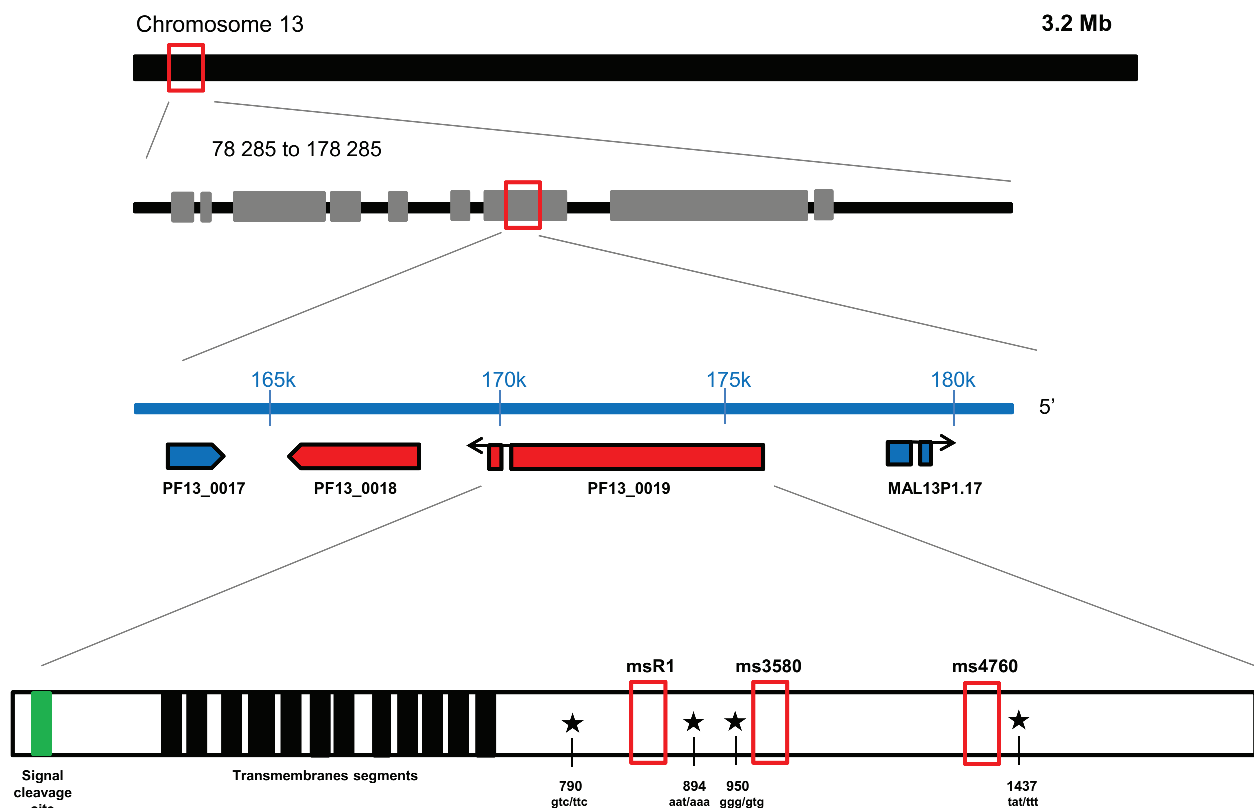

Fig. 1. Schematic representation of *pfnhe-1* gene (PF13_0019) on chromosome 13 and positions of codons polymorphisms (790, 894, 950 and 1437) and microsatellite variations (msR1, ms3580 and ms4760).

pH, volume, and ion composition are supported by transmembrane exchange of cations implying several transporters like the family of Na^+/H^+ exchangers (NHEs) (Pouyssegur et al., 1984; Putney and Barber, 2003). Investigations performed in 1993 by the group of Ginsburg have shown that the major role of *P. falciparum* Na^+/H^+ exchanger was to increase the cytosolic pH (pH_{cyt}) and to compensate acidosis caused by anaerobic glycolysis (Bosia et al., 1993). PfNHE-1, a 226 kDa protein with 12 predicted trans-membrane segments (Gardner et al., 2002; Ferdig et al., 2004), is supposed by some authors to reside in the parasite's plasma membrane (Bosia et al., 1993; Bennett et al., 2007) but others underlined that the subcellular localization of this protein is not established (Nkrumah et al., 2009). Saliba and Kirk (1999) demonstrated that *P. falciparum* maintains its pH_{cyt} by using mainly a V-type H1-ATPase, which serves as the major route for the efflux of H^+ ions (Saliba and Kirk, 1999). Later, in 2007, Bennett et al. (2007) showed that high level of QNR was correlated to an increased PfNHE-1 activity which determines pH_{cyt}. They also demonstrated that antimalarial drug resistances were related to modifications of ion transport across plasma (pH_{cyt}) and digestive vacuole (pH_{DV}) membranes and concluded that pairwise interactions of genetic determinants located on chromosome 13 and chromosome 9 affecting pH_{cyt} and PfNHE-1 were involved in QNR (Bennett et al., 2007). However, using the protocols of Bennett et al., Spillman et al. (2008) showed that the Na^+-dependent efflux of H^+ from parasites acidified using nigericin/BSA was attributable to Na^+/H^+ exchange via residual nigericin remaining in the parasite plasma membrane, rather than to endogenous transporter activity (Spillman et al., 2008). Likewise, Nkrumah et al. (2009) were unable to reproduce the Na^+/H^+-exchanger activity observed by Bennett et al., (Nkrumah et al., 2009) but they provided evidences that PfNHE-1 expression levels influenced QN sensitivity in concert with additional parasite genetic factors such as PfCRT, PfMDR1 and possibly additional yet unidentified parasite proteins. However, variations in PfNHE-1 expression levels did not impact on pH_{cyt}.

Since the seminal work by Ferdig et al. (2004), several studies have been conducted in different countries to evaluate the *pfnhe-1* polymorphisms and its association with *in vitro* QN susceptibility (Vinayak et al., 2007; Henry et al., 2009; Andriantsoanirina et al., 2010, 2012; Baliraine et al., 2010; Briolant et al., 2010, 2011; Meng et al., 2010; Okombo et al., 2010; Pelleau et al., 2011; Sinou et al., 2011). Conflicting data have been reported, likely due to the different geographical origin of parasites (implying different genetic backgrounds), the type of parasites used (fresh isolates, culture-adapted strains and reference lines) and the method used to assess *in vitro* QN susceptibility (Okombo et al., 2011; Pelleau et al., 2011). Thus, the implication of PfNHE-1 polymorphisms in QNR remains to be studied in detail.

The aim of this study was to provide a comprehensive analysis of the worldwide genetic polymorphism of ms4760 alleles of the pfnhe-1 gene and to discuss their usefulness as molecular marker of quinine resistance.

2. Plasmodium falciparum Na+/H+ exchanger (Pfnhe-1) allele polymorphism

Following the initial work of Ferdig et al. (2004) on genetic polymorphism of ms4760 within the *pfnhe-1* gene, a total of 1508 ms4760 sequences from isolates, culture-adapted parasites or reference strains from various geographical regions were retrieved from GenBank (last update on 15th June 2012) or from publications (Table 1) and were used for genetic analyses. A new numbering of ms4760 allele according to the chronological order of the data of the publication was conducted. Classification grouping ms4760 alleles according to the number of DNNND and DDNHNDNHNND repeat motifs in blocks II and V was also performed.

Ms4760 sequences were aligned and compared using the Clustal W multiple alignment algorithm in BioEdit Sequence Alignment editor (Hall, 1999). Genetic diversity was assessed by Nei's unbiased expected heterozygosity (He) from haploid data and calculated as $He = [n/(n-1)][1 - pi]$ (n = the number of isolates sampled; pi = the frequency of the ith allele) (Nei, 1978). Population genetic differentiation was measured using Wright's F statistics (Wright, 1965); population genetic parameters were computed with FSTAT software, v2.9.4 (Goudet, 1995).

The Mann-Whitney *U* test or Kruskal–Wallis method were used for non-parametric comparisons, and Student's *t* test or one-way analysis of variance for parametric comparisons. For categorical variables, Chi-squared or Fisher's exact tests were used to assess significant differences in proportions.

All reported *P*-values are two-sided and were considered statistically significant if less than 0.05.

2.1. Global genetic polymorphism of ms4760 in pfnhe-1 gene

Amongst the 1508 studied sequences, 101 different *ms4760* alleles were observed. Ms4760 alleles were renumbered according to the chronology of the publication of the studies (ranging from ms4760-1 to ms4760-101) and are presented in Table 2. Alignment of sequences of blocks I–VI in ms4760 are displayed in Fig. 2.

2.1.1. Geographical distribution of ms4760 alleles: between continents

According to the location of the sample collection, 39 ms4760 alleles were observed in Asia ($n = 398$), 74 in Africa ($n = 1070$), 5 in South America ($n = 17$), 2 in Papua New Guinea ($n = 5$) and one in Haiti ($n = 1$). Five alleles were globally distributed (ms4760-1, ms4760-3 ms4760-5, ms4760-6, ms4760-7) while others were exclusively found in Asia and in Africa ($n = 24$, ms4760-2, ms4760-8, ms4760-9, ms4760-12, ms4760-14, ms4760-15 and ms4760-18 to ms4760-35) or only in Asia ($n = 10$, ms4760-4, ms4760-10, ms4760-11, ms4760-13, ms4760-16, ms4760-17 and ms4760-98 to ms4760-101) or only in Africa ($n = 45$, from ms4760-36 to ms4760-64, from ms4760-66 to ms4760-173 & ms4760-90 to ms4760-97). Seventeen alleles have unknown origins (ms4760-65 & ms4760-74 to ms4760-89). Data are presented in Table 2 and Fig. 3.

2.1.2. Geographical distribution of ms4760 alleles: within continents and between regions

Amongst the 39 alleles found in Asia, 16 (41%) were shared between South East Asia (Thailand, Cambodia, Vietnam, Malaysia and Myanmar/China border) and Central Asia (India), whereas 23 were specific to South East Asia. In Africa, the 74 observed alleles were distributed as follows: three were shared by all regions (West Africa, East Africa, Central Africa, South Africa, Indian Ocean Islands), seven were present in all regions except South Africa, five alleles were shared by West Africa, East Africa and Central Africa, four by East Africa, Central Africa and Indian Ocean Islands and 27 by East Africa and Central Africa. Thirteen alleles were found only in Central Africa and fifteen in Indian Ocean Islands only. Among the five alleles described in South America, only two were shared by Western countries (Honduras, Colombia, Ecuador and Peru) and Eastern country (Brazil). Two were specific from Western countries and one from Eastern countries (Table 2 and Fig. 3).

2.2. ms4760 profiles: geographical distribution and prevalence

According to the number of repeats in block II (DNNND) and block V (DDNHNDNHNND) which have been associated with modulation of *in vitro* QNR (Henry et al., 2009; Andriantsoanirina et al.,

Table 1
Summarized findings of the studies describing relationships between polymorphisms in *pfnhe-1*, *pfcrt*, *pfmdr-1* and *pfmrp* genes and *in vitro* susceptibility to quinine.

References	No. and type of parasites tested	Origin of parasites	*pfnhe*–ms4760				*pfcrt* polymorphism	*pfmdr-1* polymorphism	*pfmrp* polymorphism	GenBank accession No.
			No. of alleles	Association between No. of DNNND repeats and *in vitro* QN susceptibilty	Association between No. of DDNHNDNHNND repeatsand *in vitro* QN susceptibilty	Association between ms4760 allele and *in vivo* response	Association with in vitro QN susceptibilty	Association with in vitro QN susceptibilty	Association with in vitro QN susceptibilty	
Ferdig et al. (2004)	71 *P. falciparum* lines	South-east Asia ($n = 21$), Africa ($n = 34$), Central and South America ($n = 16$)	8	IC_{90}QN: 1 repeat vs. ⩾2 repeats ($P < 0.05$ in Asia and South America clones)	NA	NA	NA	NA	NA	NA
Vinayak et al. (2007)	244 *P. falciparum* isolates	India (5 dif ferent regions)	16	NA	NA	NA	NA	NA	NA	EF123065, EF123066 DQ864466–DQ864485 EF442125–EF442130
Henry et al. (2009)	6 reference strains and 17 culture-adapted isolates	South-east Asia ($n = 5$), Africa ($n = 17$), South America ($n = 1$)	8	IC_{50}QN: 1 repeat (154 ± 110 nM) vs. 2 repeats (548 ± 253 nM) vs. 3 repeats (764 ± 332 nM). A greater number of repeat was significantly associated with increased IC_{50}QN, $P < 0.0007$)	IC_{50}QN: 1 repeat (673 ± 295 nM) vs. 2 repeats (222 ± 190 nM) vs. 3 repeats (58 nM). A greater number of repeat was significantly associated with decreased IC_{50}QN, $P = 0.002$)	NA	No significant association (M74I, N75E, K76T, A220S, Q271E/V, I356T/L, I371R), except S326D ($P = 0.0019$)	No significant association (N86Y, Y184F, S1034C, N1042D, D1246Y)	No significant association (H191Y, S437A)	NA
Andriantsoanirina et al. (2010)	83 clinical isolates	Madagascar ($n = 40$), Africa mainland ($n = 43$)	19	No significant association	IC_{50}QN: 1 repeat (1117 nM) vs. 2 repeats (192 nM). A greater number of repeat w as significantly associated with increased IC_{50}QN, $P = 0.02$)	NA	Significant association: IC_{50}QNK76 (121 nM and T76 (242 nM), $P = 0.001$	NA	NA	FJ947067–FJ947073.1
Okombo et al. (2010)	29 adapted isolates	Kenya	10	IC_{50}QN: 1 repeat (60 nM) vs. 2 repeats (227 nM) vs. 3 repeats (45 nM). The increase in the IC_{50}QN w as observed only for parasites with 2 repeats ($P < 0.05$) but not with parasites with 3 repeats ($P < 0.01$)	NA	NA	No significant association (K76T)	No significant association (N86Y), but a trend toward a decrease in IC_{50}QN in 86Y parasites (208 nM) vs. Wild-type (74 nM).	NA	HM210746–HM210771
Briolant et al. (2010)	8 reference strains and 15 culture-adapted isolates	South-east Asia ($n = 5$), Africa ($n = 15$), South America ($n = 3$)	8	No significant association	NA	NA	Significant association (M74I, N75E, K76T, A220S, I371R), except C72S, Q271E/V, I356T/L,	No significant association (N86Y, Y184F, S1034C, N1042D, D1246Y)	Significant association (H191Y, S437A)	NA
Meng et al. (2010)	60 culture-adapted	China–Myanmar	10	IC_{50}QN: 1 repeat (254 ± 89 nM) vs. 2 repeats	IC_{50}QN: 1 repeat (624 ± 337 nM) vs. 2	NA	No significant association (K76T,	No significant association (N86Y, Y184F, S1034C,	NA	NA

(*continued on next page*)

	isolates	border		(453 ± 239 nM) vs. 3 repeats (674 ± 365 nM). A greater number of repeat was significantly associated with increased IC_{50}QN ($P < 0.0045$), except for parasites with 4 repeats (462 ± 123 nM)	repeats (374 ± 244 nM). A greater number of repeat was significantly associated with decreased IC_{50}QN, $P < 0.0045$		A220S)	N1042D, D1246Y)		
Baliraine et al. (2010)	240 clinical isolates	Uganda	40	No significant association	No significant association	No significant association	NA (99.4% of 76T)	No significant association (N86Y, Y184F, D1246Y), but a trend toward a decrease in IC_{50}QN with increased number of mutations (wild-type, 65 nM; 1 mutation, 65 nM; 2 mutations, 101 nM and 3 mutations, 312 nM, $P < 0.02$)	NA	HQ412347–HQ412386
Briolant et al. (2011)	74 clinical isolates	Republic of Congo	27	No significant association	No significant association	NA	NA	NA	NA	FJ392810–FJ392827
Sinou et al. (2011)	79 clinical isolates	Vietnam	10	IC_{50}QN: 0–1 repeat (300 nM) vs. ⩾2 repeats (682 nM). A greater number of repeat was significantly associated with increased IC_{50}QN ($P = 0.015$).	IC_{50}QN: 1 repeat (704 nM) vs. 2 repeats (375 nM). A greater number of repeat was significantly associated with decreased IC_{50}QN, $P < 0.01$.	NA	NA (84% of 76T)	NA (>95% of wild-type pfmdr-1	NA	GQ845119–GQ845119–GQ465284
Pelleau et al. (2011)	90 *P. falciparum* isolates and 95 culture adapted isolates	Africa ($n = 85$), Indian Ocean ($n = 36$), Asia ($n = 38$), South America ($n = 20$), Unknown origin ($n = 2$)	32	Field isolates: no significant association. Culture-adapted isolates: IC_{50}QN: 0–1 repeat (set#1: 45 nM and set#2: 49 nM) vs. ⩾2 repeats (set#1:98 nM and set#2: 543 nM). A greater number of repeat was significantly associated with increased IC_{50}QN ($P < 0.01$).	Field isolates: no significant association. Culture adapted isolates: IC_{50}QN: For set#2, 1 repeat (557 nM) vs. 2 repeats (270 nM) vs. (118 nM) A greater number of repeat was significantly associated with decreased IC_{50}QN ($P = 0.01$).	NA	Field isolates: no significant association. Culture adapted isolates: Significant association (K76T) in set#1 (K76: 38 nM and T76: 142 nM) and set#2 (K76: 204 nM and T76: 543 nM).	Field isolates: no significant association. Culture adapted isolates: Significant association: S1034C in set#1 (S1034: 55 nM and C1042: 148 nM) and set#2 (S1034: 302 nM and C1042: 780 nM), N1042D in set#1 (N1042: 51 nM and D1042: 144 nM) and set#2 (N1042: 290 nM and D1042: 607 nM), D1246Y in set#1 (D1246: 58 nM and Y1246: 144 nM).	NA	GQ496590–GQ496601 FJ266461–FJ266471
Poyomtip et al. (2012)	85 *P. falciparum* culture-adapted isolates	Thai–Myanmar ($n = 37$) and Thai–Cambodia ($n = 48$)	?	No significant association	No significant association	NA	NA	No significant association with *pfmdr-1* copy number. Significant association: N86Y (N86: 216.5 nM and Y86: 138.3 nM, $P = 0.02$), Y184F (Y184: 160.4 nM and F184: 228.3 nM, $P = 0.01$) & N1042D (N1042: 185.8 nM and D1042: 270.5 nM, $P = 0.01$)	NA	NA
Andriantsoanirina et al. (2012)	595 *P. falciparum* isolates	Madagascar ($n = 345$), Comoros Islands ($n = 250$)	29	NA	NA	NA	NA	NA	NA	JX472441–JX472448

NA, Not available; QN, Quinine; IC_{50}, 50% inhibitory concentration; IC_{90}, 90% inhibitory concentration; Significant associations are shown in red.

Table 2
Classification of *pfnhe-1* sequences in 101 different alleles according to the geographical location of the isolates.

Continent	ASIA							AFRICA																										SOUTH AMERICA						OTHER		
Region	South East					Central	Total	West Africa													East Africa				Central Africa				South Africa		Indian Ocean Islands		Total	West				East	Total			
Country	THA	KHM	VNM	MYS	CHN/MMR	IND		MRT	SEN	GMB	MLI	GNB	SLE	LBR	CIV	GHA	TGO	BEN	BFA	NER	SDN	KEN	UGA	DJI	CAF	TCD	COG	COD	ZAF	MOZ	COM	MDG		HND	COL	ECU	PER	BRA		PNG	HTI	?
No. of isolates	8	4	81	1	60	244	398	1	5	12	21	2	2	1	5	4	1	1	4	4	6	32	241	1	3	1	76	3	2	1	255	386	1070	1	1	1	5	8	17	5	1	23
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ms4760-2																																										
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ms4760 haplotypes

Countries are designed by the 3-letters codes used by the United Nations: THA, Thailand; KHM, Cambodia; VNM, Vietnam; MYS, Malaysia; CHN, China; MMR, Myanmar; IND, India; MRT, Mauritania; SEN, Senegal; GMB, Gambia; MLI, Mali; GNB, Guinea-Bissau; SLE, Sierra Leone; LBR, Liberia; CIV, Côte d'Ivoire; GHA, Ghana; TGO, Togo; BEN, Benin; BFA, Burkina Faso, NER, Niger; SDN, Soudan; KEN, Kenya; UGA, Uganda; DJI, Djibouti; CAF, Central African Republic; TCD, Chad; COG, Congo; COD, Congo, the Republic Democratic of the; ZAF, South Africa; MOZ, Mozambique; COM, Comoros; MDG, Madagascar; HND, Honduras; COL, Colombia; ECU, Ecuador; PER, Peru; BRA, Brazil; PNG, Papua New Guinea; HTI, Haiti. Grey box means "ms-4760 haplotype never detected" and black box means "ms-4760 haplotype detected at least once".

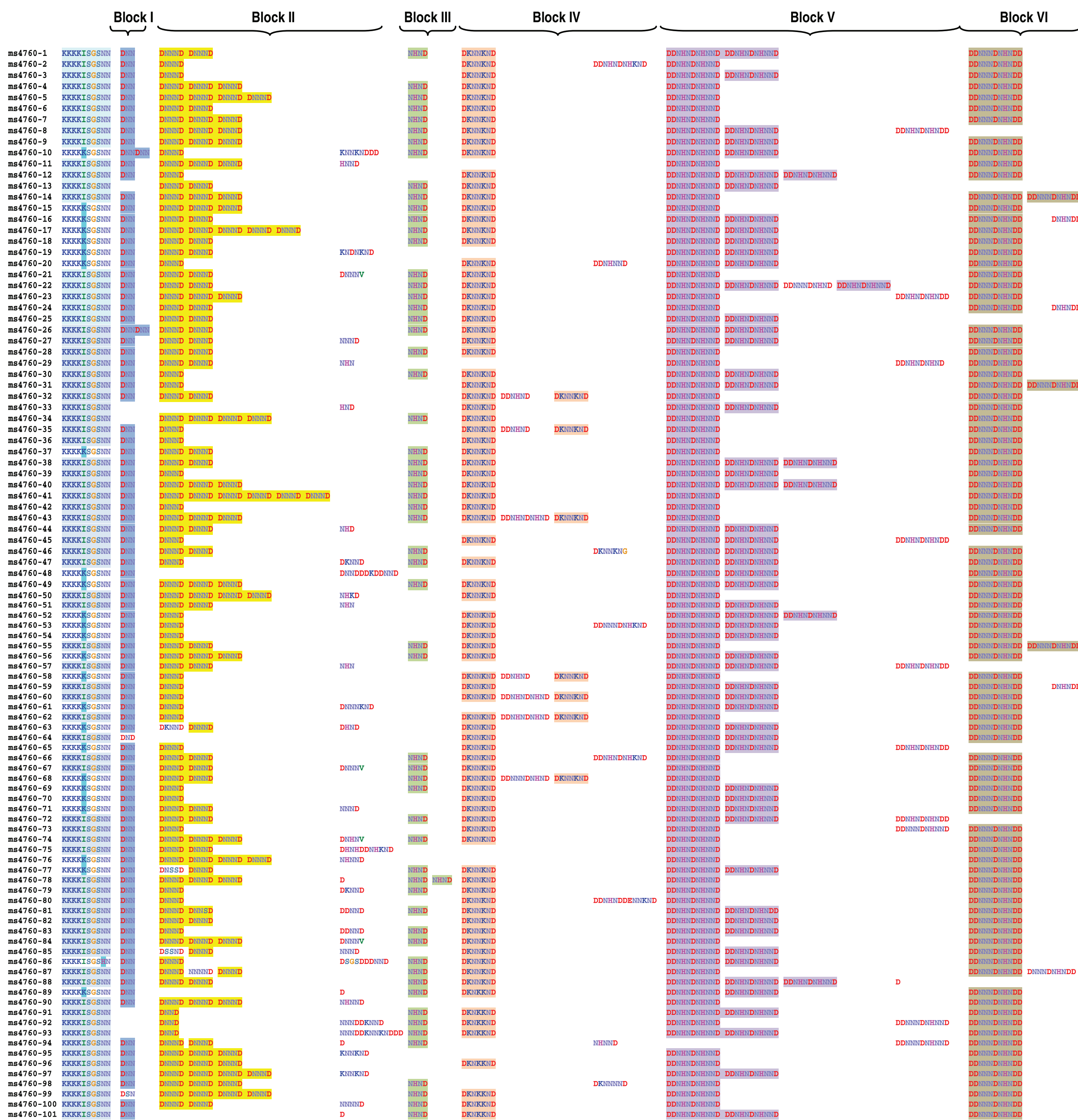

Fig. 2. Alignment of the 101 ms4760 protein sequences found in Genbank and in publications.

2010), ms4760 alleles were grouped in 15 allele profiles (from ms4760-A to ms4760-O) presented in Table 3. The number of repeats in block II (DNNND) varied from 0 (ms-4760-A) to 6 (ms4760-O) while the number of repeats in block V (DDNHNDNHNND) from 1 (ms4760-B, ms4760-E, ms4760-I, ms4760-L, ms4760-O) to 4 (ms4760-H). Sixty-six percent of ms-4760 alleles were grouped in 4 profiles: ms-4760-C [$(DNNND)_1$; $(DDNHNDNHNND)_2$] (23%), ms-4760-F [$(DNNND)_2$; $(DDNHND NHNND)_2$] (15%), ms-4760-E [$(DNNND)_2$; $(DDNHNDNHNND)_1$] (14%) and ms-4760-I [$(DNNND)_3$; $(DDNHNDNHNND)_1$] (14%). Four profiles were globally distributed (ms-4760-C, ms-4760-E, ms-4760-F and ms-4760-L), five were observed in both Asia and Africa (ms-4760-A, ms-4760-B, ms-4760-D, ms-4760-H and ms-4760-J), two were only found in Asia (ms-4760-I, ms-4760-N) and four only in Africa (ms-4760-G, ms-4760-K, ms-4760-M and ms-4760-O) (Fig. 3).

The mean number of DNNND repeats was significantly higher in Asia (2.30, SD = 0.78) compared to Africa (2.06, SD = 0.90, $P < 0.001$). Inversely, the number of DDNHNDNHNND repeats was significantly lower in Asia (1.34, SD = 0.51) compared to Africa (1.72, SD = 0.54, $P < 0.001$). Consequently, the mean ratio of DNNND/DDNHNDNHNND repeats was significantly higher in Asia (2.03 ± 0.98 vs. 1.46 ± 1.05, $P < 0.001$).

The prevalence of the *pfnhe-1* ms4760 profiles according to the geographical location of the isolates (continent & country) significantly differed between continents ($P < 0.0001$, Table 4 and Fig. 4). In both continents (Asia & Africa), 11 profiles had a low prevalence (<10%). Three profiles were predominant in Africa (ms-4760-F, 32.9%; ms-4760-C, 21.3% and ms-4760-I, 15.5%), and four in Asia (ms-4760-I, 37.1%; ms-4760-E, 28.1%; ms-4760-C, 14.3% and ms-4760-F, 10.9%). Interestingly, the prevalence of the ms-4760-C (1 DNNND repeat) decreased from Central Africa (38%) to East Africa

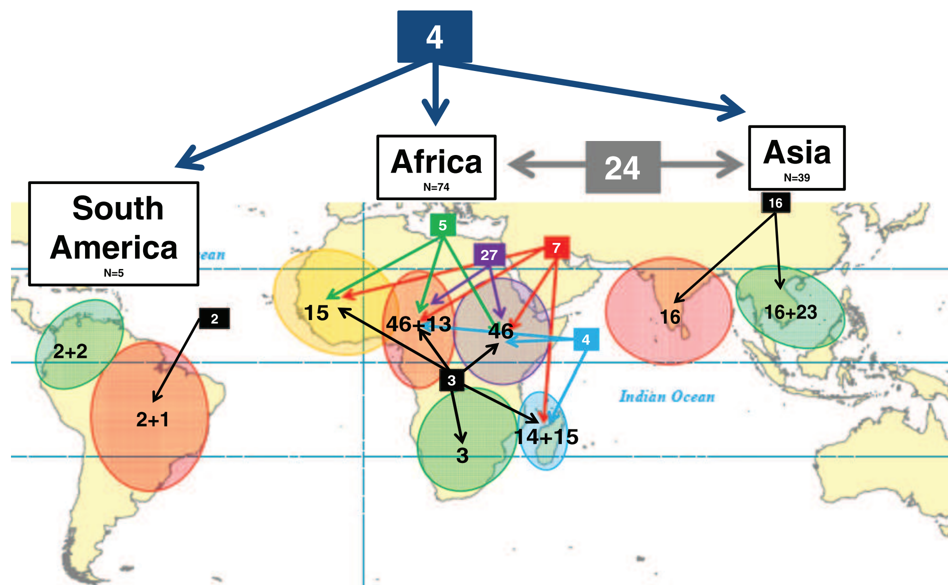

Fig. 3. Geographical distribution of the *pfnhe-1* ms4760 profile between and with continents. Numbers in boxes indicate shared ms4760 profiles between continents (blue and grey boxes) and regions (black, green, red, purple and light blue boxes). For each region, numbers of ms4760 profiles are split into shared profiles (first number) and local profiles (second number) (i.e., Madagascar: 14 shared profiles and 15 local profiles). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Phne-1 ms4760 profile groups according to the number of repeat in block II (DNNND) and block V (DDNHNDNHNND) and the geographical location of the isolates.

ms4760 alleles	ms4760 profiles	No. of haplotypes	Block II DNNND	Block V DDNHNDNHNND	Asia	Africa	South America
33, 48, 64, 89, 91 92, 93, 101	ms4760-A	8	0	2	■	■	
2, 35, 36, 42, 58, 62, 79, 80	ms4760-B	8	1	1	■	■	
3, 10, 20, 30, 31, 39, 45, 47, 53, 54, 59, 60, 61, 63, 65, 69, 70, 73, 77, 81, 83, 85, 86	ms4760-C	23	1	2	■	■	■
12, 52	ms4760-D	2	1	3	■	■	
6, 21, 24, 28, 29, 32, 37, 55, 66, 67, 68, 75, 94, 100	ms4760-E	14	2	1	■	■	■
1, 13, 16, 18, 19, 25, 26, 27, 44, 46, 51, 57, 71, 72, 82, 87	ms4760-F	16	2	2	■	■	■
38, 88	ms4760-G	2	2	3		■	
22	ms4760-H	1	2	4	■	■	
4, 7, 11, 14, 15, 23, 43, 74,78, 84, 90, 95, 96, 98	ms4760-I	14	3	1	■		
8, 9, 49, 56	ms4760-J	4	3	2	■	■	
40	ms4760-K	1	3	3		■	
5, 34, 50, 76, 99	ms4760-L	5	4	1	■	■	■
97	ms4760-M	1	4	2		■	
17	ms4760-N	1	5	2	■		
41	ms4760-O	1	6	1		■	

For each profile (A to O) and continent (Asia, Africa and South America), white box means "ms-4760 profile never detected" and black box means "ms-4760 profile detected at least once".

(21%), Indian Ocean (19%), Central Asia (19%) and South East Asia (6%) whereas the prevalence of the ms-4760-I (3 DNNND repeats) increased along the same west to east axis (7%, 14%, 17%, 23% and 61%).

2.3. Genetic diversity and genetic differentiation between parasite populations

Genetic diversity, assessed by Nei's unbiased expected heterozygosity (*He*) was significantly higher in Africa (Congo = 0.7649, Uganda = 0.7975, Kenya = 0.6582), Indian Ocean (Madagascar = 0.8053, Comoros Islands = 0.7946) or India (0.6807) compared to China/Myanmar (0.6807, $P = 0.04$) or Vietnam (0.4981, $P < 0.0001$) (Table 4).

The degree of genetic differentiation of the ms4760 profiles within parasite populations, estimated by *Fst* values, indicated a large divergence between Asian populations and African populations (Table 5). The highest differences were observed between populations from Vietnam or China/Myanmar and populations from Kenya (*Fst* = 0.319 and 0.183), Congo (*Fst* = 0.291 and 0.176), Uganda (*Fst* = 0.219 and 0.121), Madagascar (*Fst* = 0.202 and 0.111), Comoros Islands (*Fst* = 0.171 and 0.083) and India (*Fst* = 0.171 and 0.069). On the other hand, populations from Africa (Congo, Uganda, Kenya and Madagascar) showed very low divergence or were similar (*Fst* from 0.0001 to 0.076) and population from India was intermediate (*Fst* from 0.070 to 0.163).

3. Discussion

The biostatistical analyses performed in this study showed a large global genetic polymorphism of ms4760 in *pfnhe-1* gene. The African continent displayed the highest number of different alleles, followed by Asia and South America. While a few alleles were shared by three continents, others appeared restricted to Asia and/

Table 4
Prevalence and expected heterozygosity of the *pfnhe-1 ms4760* groups according to the geographical location of the isolates (continent & country)

ms4760 profiles (%)	Prevalence by Continent/Country								Total
	Africa					Asia			
	Congo	Uganda	Kenya	Madagascar	Comoros Islands	India	China/Myanmar	Vietnam	
	n = 74	*n* = 172	*n* = 29	*n* = 386	*n* = 251	*n* = 244	*n* = 60	*n* = 79	
ms4760-A	1.3	0.0	0.0	2.0	1.5	0.0	0.0	1.3	1.1
ms4760-B	4.1	1.7	7.1	4.4	1.1	0.0	1.6	0.0	2.2
ms4760-C	38.3	22.6	14.2	24.1	11.9	19.2	4.9	6.3	19.2
ms4760-D	5.4	1.1	0.0	2.3	3.5	2.8	0.0	0.0	2.3
ms4760-E	9.5	6.3	3.5	9.8	6.3	36.0	19.7	10.1	13.9
ms4760-F	26.0	33.7	53.5	31.1	34.2	10.2	14.7	10.1	26.3
ms4760-G	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1
ms4760-H	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.1
ms4760-I	6.8	13.9	14.2	14.0	21.5	23.2	50.8	69.6	21.9
ms4760-J	8.2	11.0	7.1	8.5	5.1	6.5	3.3	0.0	7.0
ms4760-K	0.0	5.2	0.0	0.0	0.0	0.0	0.0	0.0	0.7
ms4760-L	0.0	2.3	0.0	2.3	14.3	1.6	4.9	2.6	4.5
ms4760-M	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.1
ms4760-N	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.1
ms4760-O	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.1
He	0.765	0.797	0.658	0.805	0.794	0.766	0.680	0.493	

He: expected heterozygosity.

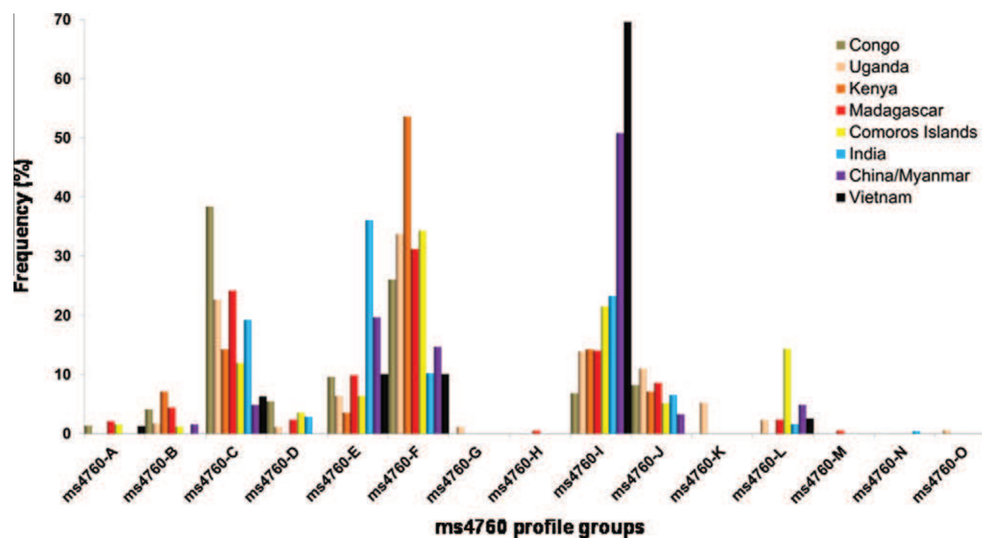

Fig. 4. Prevalence of the *pfnhe-1 ms4760* profile groups according to the geographical location of the isolates (continent and country). *Phne-1* ms4760 profile groups are described in Table 3 (according to the number of repeat in block II, DNNND and block V, DDNHNDNHNND).

Table 5
Degree of genetic differentiation of the ms4760 profile groups within parasite populations (African and Asian countries), estimated by pairwise population genetic distances (*FST*).

	Uganda	Kenya	Madagascar	Comoros Islands	India	China/Myanmar	Vietnam
Congo	0.01739	0.07610	0.01025	0.06382	0.08763	0.17689	0.29186
Uganda		0.01959	0.00011	0.01911	0.08776	0.12179	0.21948
Kenya			0.02770	0.02876	0.16355	0.18367	0.31962
Madagascar				0.02089	0.07075	0.11195	0.20280
Comoros Islands					0.09344	0.08370	0.17157
India						0.06970	0.17199
China/Myanmar							0.02694

or Africa. Similarly, within continents, some alleles were shared between various regions while others appeared restricted to specific areas as Central Africa or Indian Ocean. The geographical distribution and prevalence of ms4760 profiles, defined by variations in the number of repeats in block II and V, differed also significantly between continents. Interestingly, the mean ratio of DNNND/DDNHNDNHNND repeats, proposed by some authors as associated with *in vitro* QNR, was significantly higher in Asia than in Africa. The predominant ms4760 profiles in Africa were the same that in Asia, except for the ms-4760-E which was predominant only in Asia. Some data suggested a geographical diffusion of some alleles. The prevalence of a particular profile, the ms-4760-C (1 DNNND repeat), decreased from Central Africa to East Africa, Indian Ocean, Central Asia and South East Asia while the prevalence of another "opposite" profile, the ms-4760-I (3 DNNND repeats), increased along the same west to east axis. Genetic anal-

ysis using expected heterozygosity H_e showed a higher genetic diversity in parasites from Africa (including Indian Ocean Islands) than in those from Asia. In addition, F_{st} values indicated a large divergence between Asian and African populations. These data showed that the Asian and African populations were clearly differentiated.

Several studies have described the relationships between polymorphisms in *pfnhe-1* and *in vitro* susceptibility to quinine (Table 1). Some of these studies have also considered the association of *in vitro* susceptibility to quinine with polymorphisms in *pfcrt*, *pfmdr-1* and *pfmrp* genes, with conflicting results for *pfcrt* and *pfmrp*, and no association (except in one study) with SNPs polymorphisms of *pfmdr-1* (Table 1).

Following the inaugural study of Ferdig et al. (2004), Henry et al. (2009) investigated a series of 23 culture-adapted isolates or reference strains. The relationship between the number of DNNND repeats and the inhibitory concentration 50% values (IC_{50}) to QN was confirmed and an increased number of the DDNHNDNHNND repeat motif was associated with decreased IC_{50s} to QN. A limitation of these studies was that the *in vitro* QN susceptibility and polymorphisms determinations were performed on culture-adapted cloned isolates or reference strains, which could lead to biased results due to accumulated mutations selected by *in vitro* conditions or to selection of specific alleles during the culture. Indeed, a recent study showed an association between *pfnhe-1* polymorphism and in vitro QN response on cultured adapted isolates but not in field isolates (Pelleau et al., 2011).

An increased number of DNNND repeats was positively associated with *in vitro* QNR in six studies (Ferdig et al., 2004; Henry et al., 2009; Meng et al., 2010; Okombo et al., 2010; Pelleau et al., 2011; Sinou et al., 2011) (Table 1). All these studies used culture-adapted parasites, except study from Sinou et al. (2011). It is worth noting that Ferdig et al. (2004) used IC_{90}s rather than IC_{50}s in the other studies (Ferdig et al., 2004). Parasites having 2 or more repeats had higher IC_{90}s than parasites having 1 repeat ($P < 0.05$ for Asia and South America lines). Okombo et al. (2010) observed this association only for parasites from Kenya having 2 repeats compared to 1 repeat ($P < 0.05$) (Okombo et al., 2010). Meng et al. (2010) reported a strong positive association in a series of 60 adapted isolates from the China–Myanmar border (Meng et al., 2010). Sinou et al. (2011) also reported a positive association in a series of 51 clinical fresh isolates from Vietnam: isolates with two or more DNNND motifs were less susceptible to QN than those harbouring zero or one DNNND repeats (Sinou et al., 2011). Discordant results were reported in a Thai study by Poyomtip et al. (2012) who did not observe an association between the number of DNNND repeats and *in vitro* QNR in a series of 81 culture-adapted isolates obtained from the Thai–Myanmar border and the Thai–Cambodia border (Poyomtip et al., 2012).

Pradines and colleagues (2009–2011) published 3 studies of *pfnhe-1* polymorphisms (Henry et al., 2009; Briolant et al., 2010, 2011). In the first one, Henry et al., 2009 including 23 reference strains or culture-adapted isolates of various geographic origin, found a positive association between the number of DNNND repeats and IC_{50}s (Henry et al., 2009). In another study Briolant et al. (2010, 2011) including 23 reference strains or culture adapted isolates of similar geographic origin did not found any association (Briolant et al., 2010). Lastly, in a series of 74 clinical isolates from Republic of Congo, Briolant et al. (2011) did not found an association either (Briolant et al., 2011). Two other studies including respectively 83 and 172 clinical isolates from African countries did not find any association between an increased number of repeats in DNNND and *in vitro* QNR (Andriantsoanirina et al., 2010; Baliraine et al., 2010). The studies conducted in Asian areas reported an overrepresentation of the *ms4760*-7 allele, harboured by 49.2% and 68.3% of isolates in China–Myanmar border and Vietnam, respectively (Meng et al., 2010; Sinou et al., 2011). In the study by Meng et al. (2010), ms4760-7 isolates were among those having the lowest *in vitro* susceptibility to QN but other ms4760-7 isolates of that series displayed perfect susceptibility (Meng et al., 2010). The ms4760-7 allele was also overrepresented in the study by Henry et al. (2009) including 4 Asian isolates with high IC_{50s}, ranging from 599 to 1310 nM (Henry et al., 2009). The presence of the *ms4760*-7 allele was not rare in other areas, in particular in African countries (Ferdig et al., 2004; Andriantsoanirina et al., 2010, 2012; Okombo et al., 2010; Briolant et al., 2011) but without obvious association with QNR, many isolates harbouring this allele showing full *in vitro* susceptibility. Thus, further studies are needed to confirm whether the *ms4760*-7 allele is necessary for the emergence QN resistance and can be used in monitoring the QNR spread in South East Asia.

The number of DDNHNDNHNND repeat motif was associated with reduced *in vitro* susceptibility to QN ($P < 0.01$) in one study of 83 clinical isolates obtained in African countries (Andriantsoanirina et al., 2010). Conversely, an increased number of DDNHNDNHNND repeats was associated with higher *in vitro* susceptibility to QN in studies of isolates from the China–Myanmar border (Meng et al., 2010) or Vietnam (Sinou et al., 2011). The same association was observed in one study (Henry et al., 2009) but not confirmed in 2 subsequent studies by the same team (Briolant et al., 2010, 2011). Three other studies did not find this association either (Baliraine et al., 2010; Okombo et al., 2010; Poyomtip et al., 2012).

The usefulness of PfNHE-1 polymorphisms as marker of *in vitro* QNR may be inferred from some publications. In parasites from Asian areas, the number of DNNND repeats has been positively associated with *in vitro* QNR in culture-adapted isolates from the China–Myanmar border (Meng et al., 2010) and in isolates from Vietnam (Sinou et al., 2011) but not in culture-adapted isolates from the Thai–Myanmar border and the Thai–Cambodia border (Poyomtip et al., 2012). In parasites from Africa, the existing data show no evidence of association of the number of DNNND repeats and QN susceptibility, excluding its use as a molecular marker of QNR (Andriantsoanirina et al., 2010; Baliraine et al., 2010; Briolant et al., 2010, 2011). This may change in the future and the situation could be particular in Kenya (Okombo et al., 2010) as resistance genotypes originating from the South-East Asia may have reached this country as it was the case in the past for chloroquine and antifolates resistant *P. falciparum*.

The number of DDNHNDNHNND repeats does not seem correlated with *in vitro* QNR or contributing to QNR in Asian areas, so it does not appear as an interesting marker. Choudhary and Sharma, 2009 studied the polymorphisms in flanking microsatellites of the *pfnhe-1* gene in 108 Indian isolates (Choudhary and Sharma, 2009). They observed an expected heterozygosity of 10 flanking microsatellites in the vicinity of ±40 kb of *pfnhe-1* gene comparable to any other neutral loci. Thus, no selective sweep or valley of reduced variation around ±40 kb of this gene was observed, indicating that there was no strong selection pressure on the *pfnhe-1* gene. In addition, these authors did not find an association between DNNND repeat polymorphisms and microsatellite alleles.

The association of PfNHE-1 polymorphism and clinical resistance remains to be evaluated. Currently, only 2 cases of clinical failures have been reported. Pradines and colleagues (2011) studied a QN treatment failure in a traveller from Senegal, and observed the association of two repeats of DNNND with a reduced in vitro susceptibility ($IC_{50} = 829$ nM) (Pradines et al., 2011). The second case, a QN treatment failure in a traveller from French Guiana, did not show this association as the ms4760 microsatellite showed 1 repeat of DNNND and 2 repeats of DDNHNDNHNND, though the isolate had a reduced susceptibility to QN ($IC_{50} = 1019$ nM) (Bertaux et al., 2011).

The finding of association of polymorphisms in putative genes with clinical failures and/or *in vitro* susceptibility constitutes a pivotal step in the development of tools for the surveillance of emergence and spreading of *P. falciparum* resistant strains. Such associations must be verified on numerous isolates originating from various geographical areas and supported by molecular studies to specifically assess the involvement of the candidate genes in drug resistance. Recent genetic and physiological studies reinforced the conclusion that QNR is a complex trait requiring multiple actors (Nkrumah et al., 2009). Several transporters have been identified as determinants of resistance to quinoline antimalarial drugs. The available data on molecular surveys of potential contributors to QN resistance do not allow to propose a simple molecular typing methodology of global application. It is possibly a consequence of the multigene nature of the QNR trait, which involves multiple gene interactions. Such gene interactions depend on the alleles at play in each genetic background and likely show substantial geographic variations. In particular *pfcrt* and *pfmdr1* known to contribute to QN susceptibility have different alleles in different geographic settings (Wellems et al., 2009). For example, CQR *P. falciparum* strains have originated from at least six different geographic locations spread across Southeast Asia, Latin America and the Pacific region (Wootton et al., 2002; Wellems et al., 2009). African CQR strains have their origins in a single foundation event, a strain apparently imported from Southeast Asia. In the case of QN (and of most other antimalarial drugs), the drug pressure that selected for resistance varied considerably with respect to intensity and time in the different geographic areas. As a result, the association of different alleles of transporters with resistance to quinoline antimalarial drugs may show geographic disparities. Likewise, the amplification of *pfmdr1*, associated with *in vitro* resistance to QN, mefloquine, and halofantrine is frequent in Asia (Price et al., 2004) but rare in the African continent. Analogous processes may have occurred for *pfnhe-1*. However, the absence of selective sweep in 108 Indian *P. falciparum* isolates and the lack of association of microsatellite markers with DNNND repeats, possibly indicates that there is no strong selection pressure on the *pfnhe-1* gene (Choudhary and Sharma, 2009). Studies summarized in this paper do not exclude a potential role for PfNHE-1 in QNR in a strain-dependent manner.

In this context, the validity and reliability of candidate polymorphisms in *pfnhe-1* gene as molecular markers of QNR appears restricted to endemic areas from South Asia or possibly East African countries and needs to be confirmed.

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Conflict of interest

None declared.

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O2.2. Détection de la résistance de *P. falciparum* à l'artémisinine et développement de nouveaux outils de surveillance

Le paludisme est une maladie parasitaire, potentiellement mortelle, causée par un protozoaire du genre *Plasmodium*. Présente dans 109 pays, cette maladie est transmise à l'homme par les piqûres des moustiques femelles infestées du genre Anophèle. Parmi les cinq espèces infectant l'homme, *P. falciparum* est l'espèce la plus impliquée dans les cas de paludisme mortel (WHO 2014).

De nos jours, cette affection reste encore un problème majeur de santé publique malgré les immenses progrès accomplis depuis plus de 10 ans. Selon les dernières estimations, en 2012, 207 millions de cas de paludisme (135-287 millions) ont été enregistrés, entrainant 627 000 décès (473 000-789 000). Cette diminution spectaculaire de la mortalité (de 45% au niveau mondial par rapport à 2000 et de 49% en Afrique sub-saharienne) est due à l'intensification des efforts en matière de lutte, associée à une augmentation sans précédent des moyens financiers consentis par les organismes internationaux publics ou privés (WHO 2014). Actuellement, les stratégies de lutte s'appuient sur la prévention et la prise en charge rapide et efficace des cas de paludisme. La prévention basée sur la lutte anti-vectorielle (moustiquaires imprégnées d'insecticide à long rémanence et aspersions intra domiciliaire) vise à réduire la transmission, l'incidence/prévalence des infections plasmodiales et du paludisme clinique. La prise en charge précoce et efficace des cas (diagnostic biologique de l'infection par un examen microscopique ou par l'utilisation de tests de diagnostic rapide et traitement des cas positifs par des associations médicamenteuses à base d'artémisinine) permet de réduire la durée de la maladie, de prévenir les complications et la plupart des décès dus au paludisme. Il fait nul doute que l'efficacité de ces nouvelles combinaisons thérapeutique, associant un dérivé de l'artémisinine à action rapide avec une drogue partenaire à action plus lente ACT, Artemsinin-Combined Therapies), explique largement les résultats obtenus (WHO 2014).

La résistance aux antipaludiques est un problème récurrent. L'émergence puis la diffusion de la résistance de *P. falciparum* aux précédentes générations de médicaments comme la chloroquine et la sulfadoxine-pyriméthamine s'est généralisée au cours des années 1970 et 1980 (Mita et al. 2009). La principale conséquence a été l'anéantissant les efforts de lutte et des progrès accomplis avec une augmentation dramatique de la morbidité et de la mortalité palustre essentiellement en Afrique Sub-saharienne (Trape et al. 2002).

Depuis 2008, force est de constater que l'efficacité des ACT dans le traitement du paludisme à *P. falciparum* a diminué en Asie du Sud-Est et en particulier dans l'ouest du Cambodge (Dondorp et al. 2009). Des cas de résistance ont été observé dans de nombreuses études cliniques (Cambodge, Thaïlande, Myanmar et Vietnam) (Dondorp et al. 2009, Hien et al. 2012, Kyaw et al. 2013, Noedl et al. 2008, Phyo et al. 2012), par la mise en évidence d'une réduction importante de la vitesse d'élimination des parasites chez les patients traités (demi-vie de clairance parasitaire) par un dérivé de l'artémisinine seul ou en association avec une drogue partenaire (ACT). Bien que constituant un outil efficace pour détecter l'apparition des résistances à l'artémisinine, ces études sont logistiquement lourdes, onéreuses et donc difficiles à déployer à grande

échelle. Jusqu'à très récemment, nous ne disposions pas de test *in vitro* ni de marqueur moléculaire, outils essentiels pour étudier « directement » la résistance des parasites à l'artémisinine, ce qui entravait les efforts visant à mieux comprendre les mécanismes mis en place par le parasite pour résister à cette drogue.

Conscient que l'un des obstacles entravant à l'étude de la résistance à l'artémisinine était l'absence de tests *in vitro* permettant de détecter les souches résistantes, nous avons développé une approche originale, consistant à mimer les conditions physiologiques auxquelles les parasites étaient soumis chez l'homme. En d'autre terme, au lieu d'exposer les parasites pendant 48h à des doses sub-thérapeutiques (0.1 à 60 nM), nous avons exposé les parasites à une dose et une durée identiques à celle observé chez l'homme au cours d'un traitement par les dérivés de l'artémisinine (700 nM pendant 6 heures). Nous avons ainsi pu évaluer la différence de sensibilité *in vitro* des souches de *P. falciparum* provenant de zones où la résistance clinique à l'artémisinine était clairement observée avec celles provenant de zones idem de résistance clinique. Ce travail a permis non seulement de démontrer que cette approche était pertinente pour obtenir un phénotype *in vitro*, mais également de prouver que cette résistance était stade-dépendante (jeunes anneaux) et provoqué par un arrêt de développement des parasites exposés (dormance) **(article 5)** (Witkowski et al. 2013b). L'amélioration de ce test (appelé RSA pour Ring-stage Survival Assay) a permis de définir précisément l'âge des parasites les plus résistant au pulse d'artémisinine (stades âgés de 0-3 heures) et d'associer les données cliniques (demi-vie de clairance parasitaire) avec ce phénotype *in vitro* **(article 6)** (Witkowski et al. 2013a).

Enfin, nous avons étudié les signatures moléculaires parasitaires associées à la résistance à l'artémisinine. Pour cela nous avons comparé l'exome d'une souche d'origine africaine (F32-Tanzania), devenue résistance (F32-ART) par expositions répétées à des doses croissantes d'artémisinine pendant environ 5 ans, avec sa souche jumelle (F32-TEM) cultivée dans les mêmes conditions mais sans expositions à la drogue. Après une analyse fine de la divergence entre le génome des 2 souches, huit mutations dans sept gènes ont été identifiées. L'analyse du génome de trois souches intermédiaires (après 22, 40 et 56 cycles d'exposition) et de 49 souches isolées au Cambodge, nous a permis de déterminer la chronologie d'apparition des mutations de F32-ART et de définir une association forte entre les mutations au sein du gène PF3D7_1343700 (K13 propeller domain) et la susceptibilité *in vitro* caractérisée par le RSA. L'étude de la distribution spatiale temporelle (2001-2012) des parasites mutés, a montré un envahissement progressif des zones classées comme résistantes (l'Ouest du Cambodge), contrairement aux zones où la résistance à l'artémisinine n'avait jamais été observée (l'Est du Cambodge). Enfin, la mise en évidence de l'association entre la présence de parasites mutés dans les isolats de patients présentant une demie vie d'élimination parasitaires augmentés, a permis de confirmer que ce marqueur moléculaire était bien un déterminant majeur impliqué dans la résistance aux dérivés de l'artémisinine **(article 7)** (Ariey et al. 2014).

Article 5 : Witkowski, B., **N. Khim**, P. Chim, S. Kim, S. Ke, N. Kloeung, S. Chy, S. Duong, R. Leang, P. Ringwald, A. M. Dondorp, R. Tripura, F. Benoit-Vical, A. Berry, O. Gorgette, F. Ariey, J. C. Barale, O. Mercereau-Puijalon and D. Menard (2013). "Reduced artemisinin susceptibility of Plasmodium falciparum ring stages in western Cambodia." Antimicrob Agents Chemother **57**(2): 914-923.

Article 6 : Witkowski, B., C. Amaratunga, **N. Khim**, S. Sreng, P. Chim, S. Kim, P. Lim, S. Mao, C. Sopha, B. Sam, J. M. Anderson, S. Duong, C. M. Chuor, W. R. Taylor, S. Suon, O. Mercereau-Puijalon, R. M. Fairhurst and D. Menard (2013). "Novel phenotypic assays for the detection of artemisinin-resistant Plasmodium falciparum malaria in Cambodia: in-vitro and ex-vivo drug-response studies." Lancet Infect Dis **13**(12): 1043-1049.

Article 7 : Ariey, F., B. Witkowski, C. Amaratunga, J. Beghain, A. C. Langlois, **N. Khim**, S. Kim, V. Duru, C. Bouchier, L. Ma, P. Lim, R. Leang, S. Duong, S. Sreng, S. Suon, C. M. Chuor, D. M. Bout, S. Menard, W. O. Rogers, B. Genton, T. Fandeur, O. Miotto, P. Ringwald, J. Le Bras, A. Berry, J. C. Barale, R. M. Fairhurst, F. Benoit-Vical, O. Mercereau-Puijalon and D. Menard (2014). "A molecular marker of artemisinin-resistant Plasmodium falciparum malaria." Nature **505**(7481): 50-55.

Commentaires

Nous avons, tout d'abord, développé un nouveau test phénotypique permettant d'évaluer la résistance aux dérivés de l'artémisinine en utilisant des souches de *Plasmodium falciparum* adaptées en culture continue provenant de deux régions présentant une différence significative au niveau de la résistance aux dérivés de l'artémisinine : 10 souches de Pailin situé à l'Ouest du Cambodge et 10 souches de Ratanakiri localisé à l'Est du Cambodge, collecté entre 2010 et 2011 (Figure **12**). Les parasites ont été exposés aux stades jeunes à une dose élevée de la dihydroartémisinine pendant 6h. Les parasites prés ou post-cultures-adaptés ont été testés pour identifier leurs génotypes ou leurs clonalités.

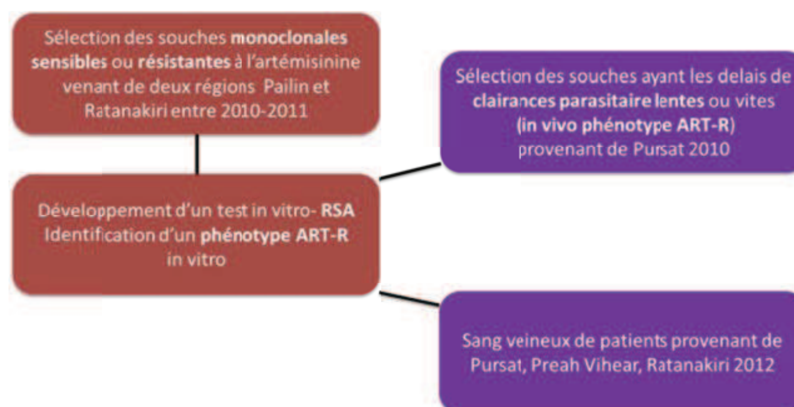

Figure 12: Stratégie d'analyse pour définir un nouveau test in vitro RSA

Dans le deuxième temps (Figure 12), nous avons amélioré le test précédent en gardant la même approche et déterminé précisément l'évolution de la survie des parasites au stade très précoce après l'exposition de la dihydroartémisinine à une dose élevée pendant 6 h sur 26 souches venant de Pursat en 2010 qui se trouve à l'Ouest du Cambodge ayant les délais de clairances parasitaires rapides ou longues (13 parasites ayant une demi-vie de clairances parasitaires rapides et 13 autres parasites ayant une demi-vie de clairances parasitaires lentes). De plus, 30 souches de terrains dans l'étude prospective dont 12 isolats venant de Pursat, 7 patients provenant de Preah Vihear et 11 cas de Ratanakiri en 2012, qui ont les données cliniques, ont été également impliquées dans le but de mesurer la durée de clairances parasitaires. Grâce aux deux précédentes études, nous avons pu caractériser le phénotype *in vitro* à l'artémisinine.

Concernant la 3ème étude (Figure 13), nous avons mis sous pression le clone F32-Tanzania (Witkowski et al. 2010) sensible à la dihydroartémisinine en cultivant en continu avec des doses croissantes de dihydroartémisinine pendant 5 ans. Nous avons pu sélectionner la souche F32-ART5 qui était devenue résistante à l'artémisinine. Les souches F32-TEM, F32-ART5 et 3D7 et 21 souches recueillis dans 10 provinces du Cambodge en 2010-2011 et adaptées en culture continue ayant leurs valeurs $RSA^{0\text{-}3h}$ ont été séquencées par la technique NGS Illumina à la Génopôle, Institut Pasteur Paris. Les analyses des polymorphismes dans 7 gènes candidats ont été étudiées, parmi lesquelles, l'identification des mutations dans un gène (PF3D7_1343700) et plus particulièrement sur le domaine propeller de la protéine putative situé sur le chromosome 13. De plus, 941 isolats sanguins entre 2001 et 2012 (Figure 14) y compris 374, 135 et 72 venant Pailin, Battambang et Pursat respectivement qui se trouvent à l'Ouest du Cambodge ; 7 et 12 provenant de Kampong Som et Kampot sucessivement qui se localisent au Sud du pays ; 32, 3 et 151 appartenant de Kratie, Mondulkiri et Ratanakiri respectivement qui se situent à l'Est du Cambodge ; et 122 et 33 venant de Preah Vihear et Oddar meanchey respectivement qui se trouvent au Nord du pays ont été utilisés pour étudier leur polymorphisme au sein de gène PF3D7_1343700 (Nested PCR puis séquençage des amplicons par la méthode Sanger).

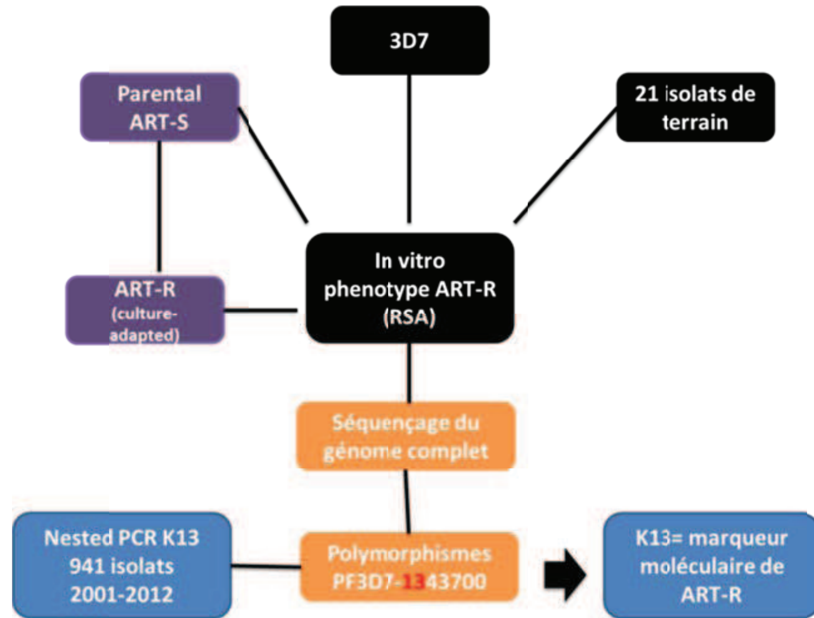

Figure 13: Stratégie utilisée pour identifier les polymorphismes du domaine propeller Kelch au sein du gène PF3D-1343700 impliqué dans l'acquisition de la résistance à l'artémisinine chez Plasmodium falciparum

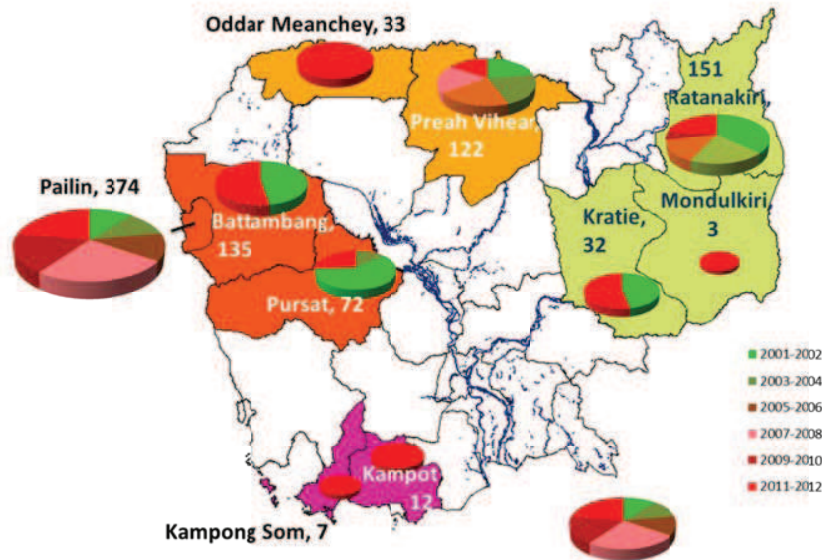

Figure 14: Etude des polymorphismes du K13 propeller dans 941 isolats cambodgiens entre 2001 et 2012

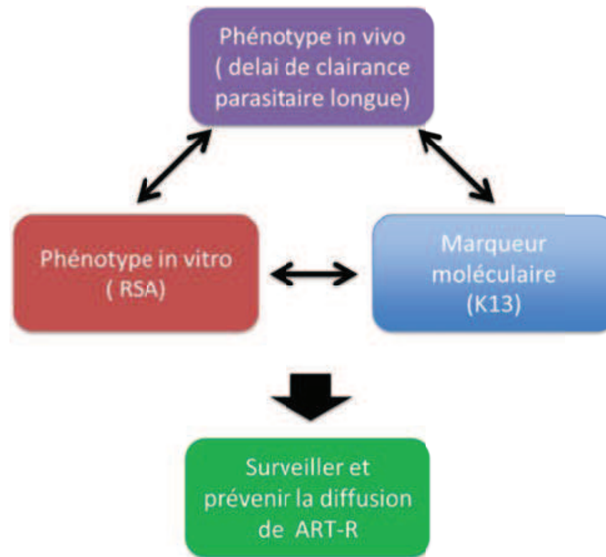

Figure 15: Approche pour surveiller et prévenir de la diffusion de la résistance à l'artémisinine chez Plasmodium falciparum

Au total 10/10 et 7/10 des isolats venant de Pailin et Rattanakiri respectivement ont été classés comme les infections monoclonales. La comparaison des profils génotypages des échantillons *ex vivo* et les souches de adaptées en culture correspondantes a montré qu'il n'y a pas de de modification des génotypes.
Aucune différence significative entre les deux groupes d'isolats n'a été observée par le test isotopic classique. Seul le RSA a permis de constater une différence de sensibilité entre les parasites au stade anneau venant de Rattanakiri et de Pailin après l'exposition à 700 nM DHA pendant 6 h. Les taux de survie parasitaires au stade anneau exposés étaient 17 fois plus élevés avec des isolats de Pailin (médiane de 13.5%) comparé à ceux de Rattanakiri (médiane de 0.8%), tandis que ceux au stade maturé exposés étaient également et très sensibles. L'amélioration de ce test (appelé RSA pour Ring-stage Survival Assay) a permis de définir précisément l'âge des parasites résistants au pulse d'artémisinine (stades âgés de 0-3 heures) et nous avons ensuite constaté que les résultats du test RSA *ex vivo* étaient significativement corrélés avec le délai de clairance parasitaire évalué par le test *in-vivo* (Witkowski et al. 2013a). La comparaison des séquences de la souche F32-Tanzania avec la souche F32-ART5 suggère que les mutations ont été acquises spécifiquement au cours de l'acquisition de la résistance à l'artémisinine. Par ailleurs, nous avons observé qu'une forte corrélation entre la présence des allèles mutants K13-propeller, le phénotype résistant à l'artémisinine dans le test *in vitro* (RSA $^{0\text{-}3h}$) et la diminution de l'efficacité des ACT dans les études cliniques (Day 3 positivity) qualifie le polymorphisme au sein du domaine K13-propeller comme un marqueur moléculaire prédictif de la résistance à l'artémisinine.

Ce travail (Witkowski et al. 2013b) a permis non seulement de démontrer que cette approche était pertinente pour obtenir un phénotype *in vitro* informatif mais également de montrer que la résistance ne concernait que le stade anneau. L'amélioration de ce test (appelé RSA pour Ring-stage Survival Assay) a permis de définir précisément l'âge des parasites résistants au pulse d'artémisinine (stades âgés de 0-3 heures) et d'associer les

données cliniques (demi-vie de clairance parasitaire) avec ce phénotype *in vitro* (Witkowski et al. 2013a). En effet, le phénotypage *in vitro* permettra de rechercher d'autres signatures moléculaires associées à la résistance à l'artémisinine. Enfin, la mise en place à large échelle d'une surveillance moléculaire basée sur la détection de mutation au sein du gène K13 à partir d'une goutte de sang par des techniques moléculaires simples et peu onéreuses, permettra d'apprécier en « temps réel », l'aire de distribution des parasites résistants et de proposer des thérapeutiques adaptées à la situation épidémiologique (Figure 15). Ce contexte, sans précédent, doit nous permettre d'éviter une nouvelle catastrophe sanitaire, telle que nous l'avons connue dans les années 80, lors de la diffusion de parasites chloroquino-résistants en Afrique. En collaboration avec l'équipe de D. Fidock, Columbia University, NY, nous avons étudié la réponse *in vitro* des parasites transfectés. Nous avons montré en particulier que l'introduction de mutations au niveau du gène K13 entraine bien une augmentation des valeurs du RSA (x70) alors que la réversion de l'allèle muté en allèle sauvage une perte de sensibilité de l'ordre de x100 (article soumis).

Article 5

Antimicrobial Agents and Chemotherapy

Reduced Artemisinin Susceptibility of Plasmodium falciparum Ring Stages in Western Cambodia

Benoit Witkowski, Nimol Khim, Pheaktra Chim, Saorin Kim, Sopheakvatey Ke, Nimol Kloeung, Sophy Chy, Socheat Duong, Rithea Leang, Pascal Ringwald, Arjen M. Dondorp, Rupam Tripura, Françoise Benoit-Vical, Antoine Berry, Olivier Gorgette, Frédéric Ariey, Jean-Christophe Barale, Odile Mercereau-Puijalon and Didier Menard
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Reduced Artemisinin Susceptibility of *Plasmodium falciparum* Ring Stages in Western Cambodia

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The declining efficacy of artemisinin derivatives against *Plasmodium falciparum* in western Cambodia is a major concern. The knowledge gap in the understanding of the mechanisms involved hampers designing monitoring tools. Here, we culture-adapted 20 isolates from Pailin and Ratanakiri (areas of artemisinin resistance and susceptibility in western and eastern Cambodia, respectively) and studied their *in vitro* response to dihydroartemisinin. No significant difference between the two sets of isolates was observed in the classical isotopic test. However, a 6-h pulse exposure to 700 nM dihydroartemisinin (ring-stage survival assay -RSA]) revealed a clear-cut geographic dichotomy. The survival rate of exposed ring-stage parasites (ring stages) was 17-fold higher in isolates from Pailin (median, 13.5%) than in those from Ratanakiri (median, 0.8%), while exposed mature stages were equally and highly susceptible (0.6% and 0.7%, respectively). Ring stages survived drug exposure by cell cycle arrest and resumed growth upon drug withdrawal. The reduced susceptibility to artemisinin in Pailin appears to be associated with an altered *in vitro* phenotype of ring stages from Pailin in the RSA.

Recent scaling up of control efforts has reduced malaria morbidity and mortality rates in many areas of endemicity (1). However, *Plasmodium falciparum* drug resistance jeopardizes these successes. Resistance to quinolines and antifolates has disseminated worldwide during the last decades (2, 3), and the situation has recently worsened, with efficacy of artemisinin (ART) derivatives declining in western Cambodia (4–6) and Thailand (7).

In western Cambodia, especially along the border with Thailand, an area considered the world's hot spot for *P. falciparum* multidrug resistance (8), artemisinin resistance (ART-R) was first suspected in 2002 to 2005, with the observation of an increased number of day 3 (D3) positive cases following treatment with artemisinin-based combination therapies, the recommended first-line treatment for uncomplicated *falciparum* malaria. The prolonged parasite clearance phenotype after treatment with artemisinin derivatives was later confirmed by clinical studies conducted in Battambang, Pailin, and Pursat, three provinces in western Cambodia, in 2006, 2008, and 2009 to 2010 (5, 6). Importantly, although evidence exists that resistance in the country is not confined to a single western province, therapeutic efficacy studies conducted in Cambodia in 2009 to 2011 by the National Center for Parasitology, Entomology and Malaria Control (CNM) indicated that ART-R has not yet emerged or spread to eastern Cambodia. The PCR-corrected treatment failure rate following dihydroartemisinin-piperaquine (DHA-PIP) treatment was approximately 25% (95% confidence interval [95% CI], 10% to 51%) in Pailin in western Cambodia and 0% in Ratanakiri in eastern Cambodia, and the proportion of patients positive on day 3 was 32.8% in Pailin but nil in Ratanakiri (9).

The declining efficacy of artemisinin derivatives is evidenced by a prolonged parasite clearance half-life (4, 7, 10). Although recent studies indicated that the phenotype of prolonged parasite clearance half-life *in vivo* is genetically determined (4, 7, 11, 12), no reliable parasite molecular marker has been identified yet. The correlation between the altered *in vivo* infection parameters and the *in vitro* drug susceptibility profile in the standard radioactive chemosensitivity assay is unclear, with a slightly elevated 50% inhibitory concentration (IC_{50}) for artemisinin derivatives in parasites collected from patients with prolonged parasite clearance times (PCTs) (5, 6) or half-life (4), but a substantial overlap in the distribution of IC_{50}s rapidly cleared parasites. To date, there has been no consensus on the mechanism of action of artemisinins or on the mechanisms by which resistance operates in the field. Mathematical modeling predicted a reduced activity of artemisinins against ring-stage parasites (ring stages) (13). *In vitro* studies with artemisinin-resistant parasites isolated from the F32-Tanzania clone showed that quiescence of ring stages was involved in survival after exposure to high doses of artemisinin (14). This was deemed not responsible for resistance by Tucker and colleagues (15), as numerous sensitive lines are able to enter a dormant state and this depends on the genetic background (16). They proposed that resistant parasites tolerate more drug by exiting dormancy and resuming growth at a greater rate than susceptible parental strains; i.e., dormant resistant parasites have a higher survival rate after dormancy (15). In a study investigating the link between dormancy and the resistance of artelinic acid (AL)-resistant para-

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sites, the AL resistance phenotype was associated with a decreased sensitivity of mature-stage parasites (mature stages), a decreased sensitivity of the ring stage to the induction of dormancy, and a faster recovery from dormancy (17). A reduced sensitivity of mature stages of ART-R laboratory lines was also reported by Cui et al. (18), who showed that resistance to artemisinin was not limited to ring-stage dormancy but also occurred in trophozoites and schizonts. This differs from the artemisinin-resistant F32-Tanzania mature stages, which are killed by artemisinin derivatives (14). How these different findings relate to the reduced susceptibility of field isolates in Cambodia is unknown.

To address this issue, we took advantage of the geographic partitioning of artemisinin resistance in Cambodia and culture-adapted *P. falciparum* field isolates from Pailin and Ratanakiri, areas of artemisinin resistance and susceptibility, respectively, and explored their *in vitro* susceptibility to dihydroartemisinin (DHA) using a panel of *in vitro* susceptibility assays. We show that ring stages from Pailin withstand artemisinin toxicity through developmental arrest or quiescence, while mature stages are fully susceptible.

MATERIALS AND METHODS

Sample collection. *P. falciparum* isolates were obtained in 2010 to 2011 from febrile patients consulting at public health centers in Pailin province (Pailin reference hospital and Ou Chra health center) and in Ratanakiri province (Veurn Say health center) (see Fig. S1 in the supplemental material and Table 1). Giemsa-stained blood smears were examined to check for *P. falciparum* monospecies infection and to evaluate parasite density as described previously (19). Samples (i) were cryopreserved in glycerolyte (20) and stored in nitrogen liquid and (ii) were processed for *in vitro* culture adaptation, and (iii) red cell pellet aliquots were stored at −20°C.

***In vitro* culture adaptation.** Culture adaptation was performed as described previously (21, 22). Briefly, after removal of plasma, the red blood cell (RBC) pellet was washed three times in RPMI 1640 supplemented with gentamicin (Gibco-Life Technologies SAS, France) and placed in culture medium (RPMI 1640, 0.5% AlbuMAX II [Gibco-Life Technologies SAS, France], 2% decomplemented B-positive [B^+] human plasma [blood bank, Phnom Penh, Cambodia]) at 4% hematocrit at 37°C in 5% O_2–5% CO_2–90% N_2. Parasitemia, checked daily, was kept below 2% by addition of fresh blood (blood bank, Phnom Penh, Cambodia).

Culture adaptation was considered successful after 3 weeks of uninterrupted culture for parasites that withstood two successive rounds of cryopreservation/thawing. Parasites used here were cultured after the third cryopreservation/thawing procedure.

Laboratory reference clones from the Malaria Research and Reference Reagent Resource Center (MR4) (MRA-102 [strain 3D7], MRA-157 [W2], MRA-152 [7G8], and MRA-155 [HB3]; ATCC, Manassas, VA) and the wWARN *in vitro* module (G15) were cultivated under the same conditions.

Multiplicity of infection (clonality). Parasite DNA was extracted from fresh blood collected at h 0 (H0) before antimalarial treatment administration and from the matched culture-adapted isolates using a QIAamp DNA blood minikit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. Genotyping was carried out using allelic family-specific nested PCR (MAD20, K1, and RO33 for *msp-1* and 3D7 and FC27 for *msp-2* and *glurp*), as described previously (23). Paired *ex vivo*/culture-adapted isolates were analyzed on the same days and on the same agarose gels.

The multiplicity of infection (clonality) was calculated from the highest number of alleles detected at any of the three loci.

All PCR amplifications contained a positive control (genomic DNA from strains W2, HB3, and 3D7 Africa) and a negative control (no-target DNA).

Quantitative *msp-1* PCR. For the quantitative PCR targeting *msp-1* (24), DNA extracts before exposure and daily from D1 to D6 were used. PCRs were carried out in 20-μl volumes in a 96-well plate containing 1× HOT FIREPol EvaGreen qPCR mix (Solis Biodyne, Taru, Estonia), 0.5 μM (each) primer (for K1, 3′-gaaattactacaaaaggtgcaagtg-5′ and 3′-agatgaagtatttgaacgaggtaaagtg-5′; for Mad20, 3′-gaacaagtsgaacagctgtta-5′ and 3′-tgaattatctgaaggatttgtacgtcttga-5′), and 2 μl of template DNA.

Amplifications were performed under conditions of 94°C for 15 min and 45 cycles of 94°C for 15 s, 59°C for 90 s, and 72°C for 60 s, followed by melt analysis (from 65°C to 90°C) to check the specificity of the PCR products. Detection of Mad20 and K1 *msp-1* alleles was performed using a LightCycler 480 system (Roche Diagnostics, Basel, Switzerland).

Results were analyzed by the comparative threshold cycle (C_T) method (25), based on the finding that the Mad20 and K1 alleles amplified with the same efficiency over a range of DNA concentrations. Differences in C_T (ΔCt) values of Mad20 and K1 alleles in exposed and nonexposed cultures were calculated as follows: $\Delta Ct = Ct_{Mad20} - Ct_{K1}$. The change in ΔCt over time was computed as $\Delta\Delta Ct_{t(n)} = \Delta Ct_{t(n)} - \Delta Ct_{t(0)}$, where n is time (in days) and $t(0)$ is day 0. Relative expression levels were then calculated as $2{-}\delta\delta Ct_{t(n)}$ to account for the exponential properties of PCR. All reactions were performed in triplicate.

Experimental *in vitro* assays (Fig. 1). (i) Standard *in vitro* drug susceptibility assay (SIA). Parasite sensitivity to eight antimalarial drugs was assessed using the standard isotopic 48-h test (26) and 3D7 as the reference strain. Artesunate, dihydroartemisinin, doxycycline, chloroquine, mefloquine, piperaquine, and quinine were obtained from the WWARN QA/QC module (www.wwarn.org/research/tools/qaqc) and atovaquone from Sigma-Aldrich (Singapore). Stock solutions of antimalarial drugs were prepared in water (Biosedra, France) for chloroquine, 0.5% lactic acid for piperaquine, and methanol for the other drugs. The final plate concentrations ranged from 0.1 to 102.4 nM for artesunate, 0.0625 to 64 nM for dihydroartemisinin, 5 to 5,120 nM for chloroquine, 1 to 1,024 nM for mefloquine, 2 to 2,000 nM for piperaquine, 6.25 to 6,400 nM for quinine, 0.1 to 100 nM for atovaquone, and 1 to 500 μM for doxycycline.

Results were expressed as the 50% inhibitory concentration (IC_{50}), defined as the concentration at which 50% of [^{3}H]hypoxanthine incorporation was inhibited compared to the drug-free control well results, and determined by nonlinear regression using online ICEstimator software (www.antimalarial-icestimator.net).

(ii) Ring-stage survival assay (RSA) and mature-stage survival assay (MSA). Parasite cultures were synchronized by three 5% sorbitol treatments (27). Parasites at the ring or mature stage (2 to 12 h and 24 to 36 h postreinvasion for the ring and mature stages, respectively) at 1% to 2% parasitemia, 2% hematocrit, and a 2-ml final volume were exposed for 6 h to 700 nM DHA or 0.1% dimethyl sulfoxide (DMSO) for the control cultures, washed, and resuspended in drug-free culture medium.

In the RSA and the MSA, susceptibility to DHA was assessed microscopically on thin films by estimating the percentage of viable parasites that had developed into a second generation of rings or trophozoites at 66 h and 42 h following exposure of ring- or mature-stage parasites, respectively (see Fig. 1).

The interassay repeatability of the RSA was assessed for 13 adapted lines on successive batches of cultures from the same thawed cryopreserved sample.

(iii) Recovery assay (RA). A phenotype used in previous work with reference lines was time to recovery to initial parasitemia (14) or time to growth recovery, monitoring parasite counts for 5 days to several weeks postexposure (15, 16, 18). We monitored time to initial parasitemia after a 6-h 700 nM DHA pulse in the recovery assay. Giemsa-stained blood smears were made every 24 h after drug exposure for 6 consecutive days and examined by two independent microscopists. Recovery was expressed as the percentage of initial parasite density.

In mixed-culture experiments, synchronous ring-stage parasites with different levels of susceptibility in the RSA and distinct *msp-1* alleles (PL4971 from Pailin and RT4974 from Ratanakiri) were mixed at a 1:1

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TABLE 1 Characteristics of patients and sample isolates, Cambodia, 2010 to 2011[a]

										Genotyping result (at D0 before antimalarial treatment and after culture adaptation) for *ex vivo*-adapted/culture-adapted isolates					
IPC ID	Field ID	Mo of collection	Site	Project name	Age (yr)	Sex	D0 parasite density	Treatment received	MOI	Mad20	K1	RO33	3D7	FC27	*glurp*
4248	KH004_003	May 2011	Pailin	TRAC	19	Female	103,620	AS*3 + DHA/PIP	1	230				400	630
4970	PLPF004	September 2011	Pailin	WHO	15	Male	17,100	A_M	1	180				400	550
4971	PLPF005	September 2011	Pailin	WHO	32	Male	9,234	A_M	1	120			300		450
4992	PLPF006	September 2011	Pailin	WHO	30	Male	165,781	A_M	1	150			300		750
5035	KH004_030	September 2011	Pailin	TRAC	39	Male	63,554	AS*3 + DHA/PIP	1	180				380	750
5100	KH004_033	September 2011	Pailin	TRAC	46	Female	31,651	AS*3 + DHA/PIP	1	180				380	750
5145	PLPF008	September 2011	Pailin	WHO	30	Male	227,524	A_M	1	120			300		750
5160	KH004_034	September 2011	Pailin	TRAC	25	Male	57,776	AS*3 + DHA/PIP	1	230				400	630
5168	KH004_035	September 2011	Pailin	TRAC	24	Male	40,192	AS*3 + DHA/PIP	1	150			350		550
5208	PLPF009	October 2011	Pailin	WHO	38	Male	51,406	A_M	1	120				400	750
3592	RTKPF033	November 2010	Ratanakiri	WHO	15	Male	20,378	AM_LUM	>1	200	160		320	320	680/750
4880	04PF026	August 2011	Ratanakiri	Sentinel Network	20	Male	3,585	A_M	>1	200	160		280		680
4914	04PF029	August 2011	Ratanakiri	Sentinel Network	14	Male	65,820	A_M	1	180			350		580
4974	04PF033	September 2011	Ratanakiri	Sentinel Network	25	Female	15,358	A_M	1		160		420		580
5055	04PF035	September 2011	Ratanakiri	Sentinel Network	46	Male	47,960	A_M	>1	230/300			320	400	600/630
5150	04PF038	September 2011	Ratanakiri	Sentinel Network	5	Male	35,629	A_M	1	230			320		680
5152	04PF040	September 2011	Ratanakiri	Sentinel Network	7	Female	23,540	A_M	1	150			320		680
5159	04PF041	September 2011	Ratanakiri	Sentinel Network	20	Female	55,100	A_M	1	150			320		750
5188	04PF042	October 2011	Ratanakiri	Sentinel Network	23	Male	2,530	A_M	1	120			250		750
5207	04PF043	October 2011	Ratanakiri	Sentinel Network	35	Male	5,318	A_M	1	200				470	680

[a] ID IPC, Institut Pasteur in Cambodia identification number; MOI, multiplicity of infection. AS*3 + DHA/PIP: D0, artesunate at 4 mg/kg of body weight; D1, artesunate at 4 mg/kg; D2, artesunate at 4 mg/kg; D3, DHA/PIP (40 mg DHA plus 320 mg piperaquine) (Duo-cotecxin; Zhejiang Holley Nanhu Pharamaceutical Co. Ltd., Jiaxing City, China), 3 tablets; D4, DHA/PIP, 3 tablets; D5, DHA/PIP, 3 tablets. AM_LUM: D0, artemether at 20 mg and lumefantrine at 120 mg (Coartem; Novartis, Bale, Switzerland), 8 tablets; D1, 8 tablets; D2, 8 tablets. A_M: D0, artesunate at 12 mg/kg (Guilin, Shanghai, China) and mefloquine at 25 mg/kg (AECH, Basel, Switzerland); D1, artesunate at 12 mg/kg and mefloquine at 25 mg/kg; D2, artesunate at 12 mg/kg and mefloquine at 25 mg/kg. Parasite density is expressed as the number of parasites per microliter. NA, not available. Parasite isolates were selected according to the following criteria: date of collection and successful adaption to *in vitro* culture.

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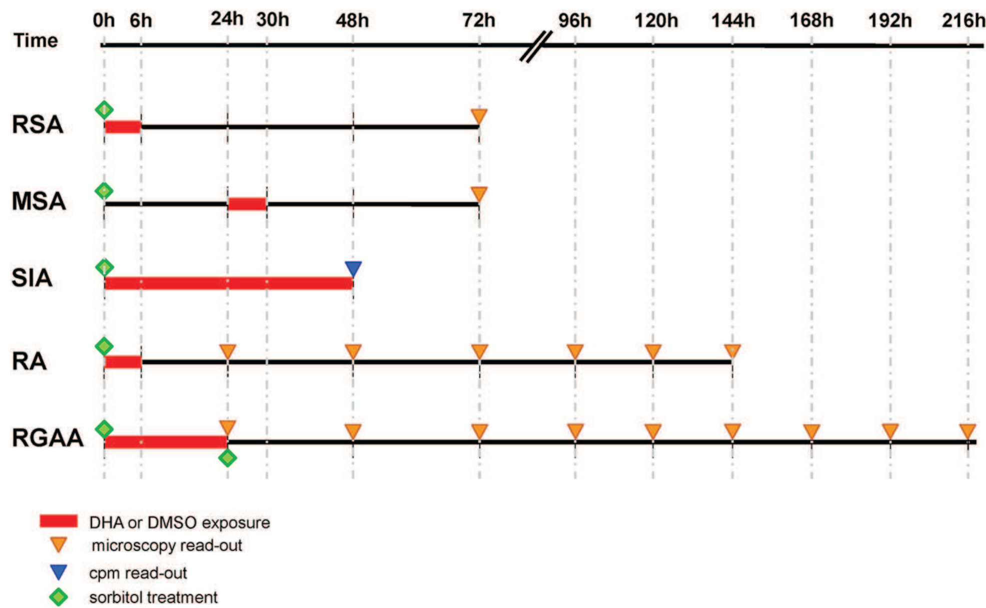

FIG 1 Schematic representation of the various *in vitro* phenotypes investigated with synchronous cultures. RSA, ring-stage survival assay; MSA, mature-stage survival assay; SIA, standard *in vitro* drug susceptibility assay; RA, recovery assay: RGAA, ring-stage growth arrest assay.

ratio or cultivated separately, exposed for 6 h to 700 nM DHA, and cultivated for 6 days in the absence of drug.

(iv) Ring-stage growth arrest assay (RGAA). Synchronized ring stages (1 to 2% parasitemia, 2% hematocrit, 10 ml final volume) were exposed for 24 h to 700 nM DHA or to 0.1% DMSO in the case of control cultures. After drug exposure, cultures were split in two flasks, and one was treated with 5% sorbitol to lyse mature stages (27). Cultures were resuspended in culture medium, and parasitemia was monitored by microscopy every day until day 10. Results were expressed as percentages of initial parasite density.

Statistical analysis. Microsoft Excel software (Microsoft Office 2010) and MedCalc software (version 12; Mariakerke, Belgium) were used for data analysis. Data from the standard *in vitro* assays were analyzed after logarithmic transformation and expressed as medians with 95% confidence intervals. Categorical variables were compared by using the chi-square test or Fisher's exact test and continuous variables by using the Mann-Whitney *U* test. The degree of association between two variables was calculated using Spearman's correlation coefficient.

The interassay repeatability of the RSA was calculated using the average, the standard deviation (SD), and the relative standard deviation (RSD) (RSD = 100 × [SD/average], expressed as a percentage).

P values < 0.05 were considered to indicate statistically significant differences.

Ethical statement. Ethical clearances for collection of patients' isolates were obtained from the Cambodian National Ethic Committee for Health Research (NECHR; institutional review board [IRB] no. 160, 28 October 2010, and IRB no. 007, 14 February 2011) and the Technical Review Group of the WHO Regional Office for the Western Pacific (no. ACTRN126000181808).

Informed written consent was provided by all patients or their parents or guardians before inclusion in the study.

RESULTS

Twenty isolates collected from patients with pure *P. falciparum* infection enrolled in clinical efficacy trials (10 patients from Pailin and 1 from Ratanakiri) or consulting in health centers (9 patients from Ratanakiri) were successfully adapted for long-term culture. Overall, 10/10 and 7/10 isolates from Pailin and Ratanakiri, respectively, were classified as monoclonal infections. Comparison of the genotyping profiles of the *ex vivo* sample and the respective culture-adapted line showed that there was no loss or modification of genotypes. We concluded that culture adaptation did not modify the genotyping pattern and hence clonality of the isolates studied (Table 1).

In the standard growth inhibition assay (SIA), the median values of the IC_{50} for artemisinin derivatives (AS and DHA), mefloquine, piperaquine, quinine, and doxycyclin were similar in the isolates from Pailin and Ratanakiri. However, the isolates from Pailin had a higher median IC_{50} for chloroquine than those from Ratanakiri (170 nM and 69 nM, respectively) ($P = 0.01$) and tended to have a lower median IC_{50} for atovaquone (6.9 nM and 11.2 nM, respectively) ($P = 0.02$) (Fig. 2).

***In vitro* phenotype of reduced susceptibility to artemisinin derivatives.** We next explored the susceptibility of synchronous cultures of mature stages and ring stages to a 6-h pulse with 700 nM DHA, a physiologically relevant, high drug dose, in the MSA and RSA, respectively, and explored survival of the parasites (Fig. 1; see also Fig. S2 in the supplemental material).

The MSA showed that mature stages were fully susceptible, as more than 99% of the parasites were recorded as dead within 42 h following drug exposure. The high susceptibility of mature stages was observed for all parasite lines, irrespective of their geographic origin (Fig. 3). At 72 h, the median percentages of parasites in drug-exposed Pailin and Ratanakiri isolates were similar (medians, 0.6% and 0.7%, respectively; $P = 0.76$).

In contrast, the RSA identified marked differences between ring stages from Pailin and Ratanakiri after exposure to a 6-h pulse of 700 nM DHA (Fig. 3). As a consequence, parasite viability was assessed at 72 h, i.e., after erythrocyte invasion and initiation of the subsequent erythrocytic cycle. The median percentage of viable parasites at 72 h was significantly higher ($P = 0.0002$) for parasites from Pailin (13.5% [95% CI, 4.9% to 17.0%; range, 3.9% to 19.7%]) than for parasites from Ratanakiri (0.8% [95% CI, 0.2%

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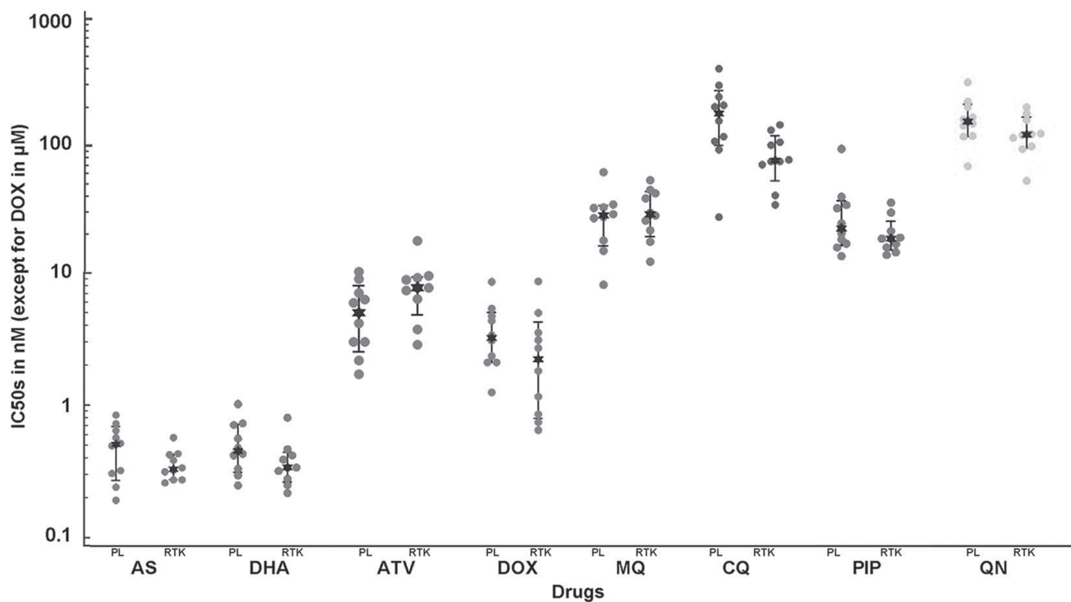

FIG 2 *In vitro* susceptibility to eight antimalarial drugs (expressed as IC_{50}) of 20 adapted parasites collected in Pailin ($n = 10$) and Ratanakiri ($n = 10$) in 2010 to 2011 determined using the standard growth inhibition assay (SIA). PL, Pailin; RTK, Ratanakiri. Median IC_{50}s: for artesunate (AS), 0.61 nM for PL and 0.38 nM for RTK ($P = 0.26$); for dihydroartemisinin (DHA), 0.47 nM for PL and 0.32 nM for RTK ($P = 0.07$); for atovaquone (ATV), 6.9 nM for PL and 11.2 nM for RTK ($P = 0.02$); for doxycycline (DOX), 5.7 µM for PL for PL and 4.8 µM for RTK ($P = 0.22$); for mefloquine (MQ), 30.4 nM for PL and 31.6 nM for RTK ($P = 0.60$); for chloroquine (CQ), 170 nM for PL and 69 nM for RTK ($P = 0.01$); for piperaquine (PIP), 37.6 nM for PL and 33.2 nM for RTK ($P = 0.10$); and for quinine (QN), 198 nM for PL and 160 nM for RTK ($P = 0.25$). The quality control data determined using 3D7 were as follows (median IC_{50} ± SD): 0.7 nM ± 0.3 nM ($n = 21$) for AS, 0.5 nM ± 0.2 nM ($n = 21$) for DHA, 19 nM ± 9 nM ($n = 22$) for ATV, 5.21 µM ± 1.8 nM ($n = 21$) for DOX, 24 nM ± 9 nM ($n = 21$) for MQ, 16 nM ± 4 nM ($n = 22$) for CQ, 36 nM ± 5 nM ($n = 22$) for PIP, and 74 nM ± 32 nM (N=22) for ON.

to 0.9%; range, 0.2% to 3.3%]) or laboratory reference clones (2.0% [range, 0.6% to 5.4%]) (Table 2).

The mean standard deviation (SD) and the mean relative standard deviation (RSD) of the RSA interassay repeatability ($n = 13$) were estimated as 1.8% (95% CI, 0.7% to 3.0%) and 22% (95% CI, 16% to 28%), respectively. Variations in parasite density evaluations assessed by microscopy readings were higher for exposed cultures (mean RSD, 34% [95% CI, 25% to 43%]) than for non-exposed cultures (mean RSD, 8% [95% CI, 6% to 9%]) (see Table S1 in the supplemental material).

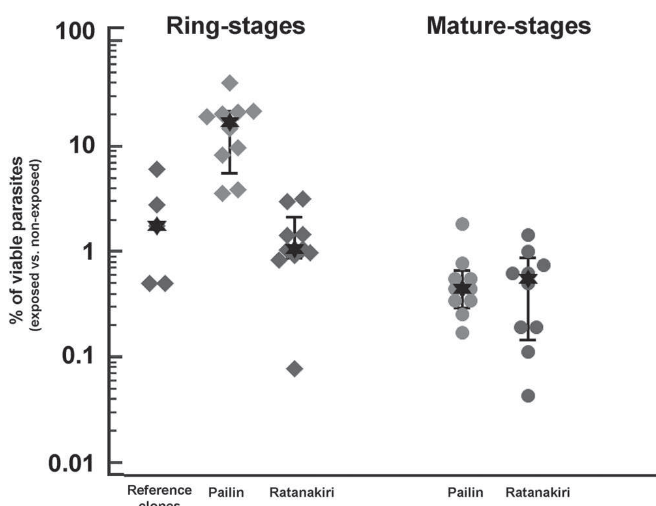

FIG 3 Results of the RSA and MSA expressed as the percentage of viable parasites following a 6-h exposure to 700 nM DHA of ring- and mature-stage parasites from Pailin and Ratanakiri and reference clones (W2, 3D7, HB3, G15, and 7G8).

In addition, no correlation was found between the RSA and MSA or between the RSA and IC_{50}s for DHA in any parasite line.

Recovery to initial parasitemia level. Recovery to the initial level of parasitemia after the 6-h 700 nM DHA pulse (RA; see Fig. 1) was monitored for two isolates, RT4974 (from Ratanakiri) and PL4971 (from Pailin). Figure 4A shows that RT4974 parasitemia dropped quickly to very low levels (<5% of initial parasitemia) and remained so for the next 5 days. In contrast, PL4971 parasitemia dropped more slowly during the first 2 days and rebounded thereafter, such that the initial values were reached again between day 4 and day 5. Overall, for the set of six isolates studied, significant correlations were observed between the percentages of viable parasites in the RSA and in the recovery assay monitored at D6 ($n = 6$, $r = 0.95$ [95% CI, 0.61 to 0.99; $P = 0.003$]) and at D10 ($n = 6$, $r = 0.83$ [95% CI, 0.06 to 0.98; $P = 0.04$]) (Table 2).

To further document the *in vitro* phenotype of quicker (more efficient) recovery of ring stages from Pailin parasites to a 6-h exposure to 700 nM DHA, we performed a RT4974 plus PL4971 mixed-culture experiment. Recovery of the mixed culture matched the calculated 50% survival of the individual cultures (Fig. 4A). This was further documented by quantitative genotyping of the cultures. As PL4971 and RT4974 harbor distinct *msp-1* block 2 alleles, a Mad20-type and a K1-type allele, respectively, we monitored the allele ratio during the next 6 days of cultivation. Whereas the 1/1 ratio of Mad20 to K1 *msp-1 block2* alleles was stable in the mixed unexposed culture, there was a progressive increase in the level of the Mad20 allele harbored by PL4971 in the mixed drug-treated culture, consistent

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TABLE 2 *In vitro* phenotype of culture-adapted parasites from Pailin and Ratanakiri collected in 2010 to 2011 and reference clones determined using the standard *in vitro* drug susceptibility assay, the ring-stage survival assay, and the recovery assay at D3, D6, and D10[a]

		SIA IC_{50} (nM)			RA (%)		
IPC ID	Site	AS	DHA	RSA (%)	D3	D6	D10
5145	Pailin	0.7	0.77	14.2	1.70	3.78	47.83
4970	Pailin	0.8	0.34	15.5	NA	NA	NA
4992	Pailin	0.35	0.5	13.8	0.0	2.5	14.54
4248	Pailin	0.27	0.25	7.1	NA	NA	NA
5035	Pailin	2.92	4.79	7.1	NA	NA	NA
5100	Pailin	0.63	0.44	4.2	NA	NA	NA
5160	Pailin	0.21	0.3	19.6	NA	NA	NA
5168	Pailin	0.37	0.45	14.2	NA	NA	NA
5208	Pailin	0.63	0.6	3.9	NA	NA	NA
4971	Pailin	1.08	1.13	17.4	0.87	3.37	77.71
5188	Ratanakiri	0.31	0.2	3.3	NA	NA	NA
3592	Ratanakiri	1.1	1,00	1.6	0.0	0.88	10.66
5150	Ratanakiri	0.51	0.45	0.7	0.0	0,0	2.5
5055	Ratanakiri	0.31	0.32	0.16	NA	NA	NA
4974	Ratanakiri	0.5	0.23	1.3	0.0	0.0	3.48
5152	Ratanakiri	0.29	0.37	0.6	NA	NA	NA
5159	Ratanakiri	0.45	0.4	0.6	NA	NA	NA
4880	Ratanakiri	0.39	0.26	1.3	NA	NA	NA
5207	Ratanakiri	0.36	0.3	1.1	NA	NA	NA
4914	Ratanakiri	0.37	0.32	2.5	NA	NA	NA
3D7	Reference clone	0.58	0.47	0.8	NA	NA	NA
G15	Reference clone	NA	NA	3.5	NA	NA	NA
W2	Reference clone	0.71	0.63	5.4	NA	NA	NA
7G8	Reference clone	NA	NA	1.7	NA	NA	NA
HB3	Reference clone	NA	NA	0.6	NA	NA	NA

[a] SIA, standard *in vitro* drug susceptibility assay; RSA, ring-stage survival assay; RA, recovery assay; AS, artesunate; DHA, dihydroartemisinin; IC_{50}, 50% inhibitory concentration; RSA (%), percentage of viable parasites having developed into a second generation of rings or trophozoites at 66 h following exposure of ring stages; RA (%), percentage of recovery from the initial parasitemia.

with a substantial loss of the RT4974 parasites after drug treatment (Fig. 4B).

Reduced ring-stage susceptibility to DHA and developmental arrest. To substantiate that the reduced susceptibility to DHA of Pailin isolates in the RSA was mediated by developmental arrest at the ring stage, we extended the duration of the 700 nM DHA exposure to 24 h and carried out a sorbitol treatment immediately thereafter in order to eliminate any parasite(s) that had matured during drug exposure.

In the cultures that were not exposed to DHA, the 24-h incubation allowed maturation of the parasites and, as predicted, sorbitol treatment induced a massive infected red cell lysis and a dramatic drop of parasite counts (Fig. 5A). In contrast, sorbitol lysis had no impact on the growth curve of DHA-treated cultures, indicating developmental arrest of ring stages and minimal sorbitol-induced loss of mature parasites (Fig. 5B).

Examination of Giemsa-stained smears of DHA-exposed parasites showed that many ring stages became pyknotic within 24 h, while the surviving ring stages remained morphologically unaltered and apparently blocked in their development as they resumed growth thereafter. This is detailed in Fig. 6. In the control nonexposed culture (upper panels), viable parasites were detected at H24 (trophozoites), at H48 (schizonts/newly invaded rings), and at H72 (trophozoites). The lower panels of the figure illustrate the morphology of DHA-exposed parasites and show a typical example of doubly infected RBCs, with one surviving parasite displaying a normal morphology (H24 rings, H48 trophozoites, and H72 schizonts) and a second pyknotic intracellular parasite.

DISCUSSION

A better understanding of the mechanisms allowing parasites to withstand artemisinin toxicity in western Cambodia is urgently needed. We used here culture-adapted parasites originating from two geographically distant areas of Cambodia, Pailin, where *in vivo* artemisinin resistance is established and commonly observed, and Ratanakiri, where there is no sign of reduced artemisinin efficacy (9). We examined susceptibility to DHA using several *in vitro* assays in order to identify a robust test that would differentiate parasites from the two areas.

There was no evidence of differing susceptibilities to artemisinins among the various lines in the standard *in vitro* growth inhibition assay. Importantly, mature stages appeared equally susceptible to a 6-h pulse with 700 nM DHA in the two sets of cell lines (Fig. 3). However, ring-stage susceptibility in the RSA differed markedly between parasites from Pailin and Ratanakiri (Fig. 3 and Table 2). The Pailin parasite lines consistently displayed much higher survival rates (an approximately 17-fold increase) at 72 h than parasites from Ratanakiri or reference lines. Follow-up over a 6-day period (RA) showed a reduced initial slope of decaying parasitemia in lines with a high percentage of viable parasites in RSA compared to the slope seen with lines with a low percent-

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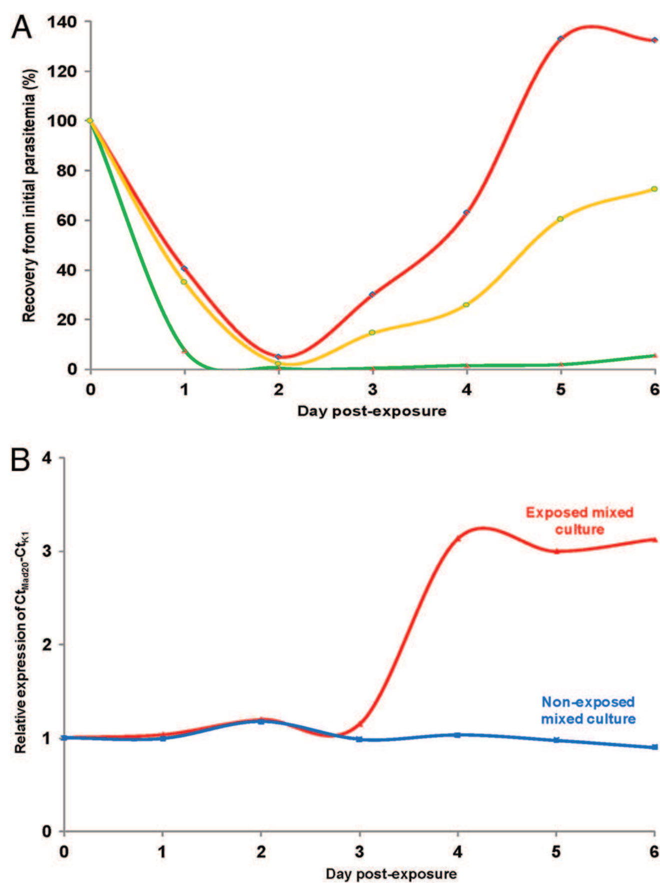

FIG 4 Results of the recovery assay (RA) following a 6-h exposure to 700 nM DHA of ring-stage parasites in separate and mixed cultures. (A) Recovery of the initial parasitemia for PL4971 from Pailin (red), RT4974 from Ratanakiri (green), and a mixed culture with an initial 1:1 ratio (orange). Recovery of the initial parasitemia from D2 to D6 of separate cultures (for PL4971, 5%, 30%, 63%, 133%, and 132.5%; for RT4974, 0.45%, 0.3%, 1.4%, 1.9%, and 5.5%) and mixed cultures (2.35%, 14.5%, 26%, 60.4%, and 72.5%) that were perfectly matched. (B) Relative expression of *msp-1* alleles (Ct_{Mad20} for PL4971 and Ct_{K1} for RT4974) in exposed cultures (red) and nonexposed cultures (blue).

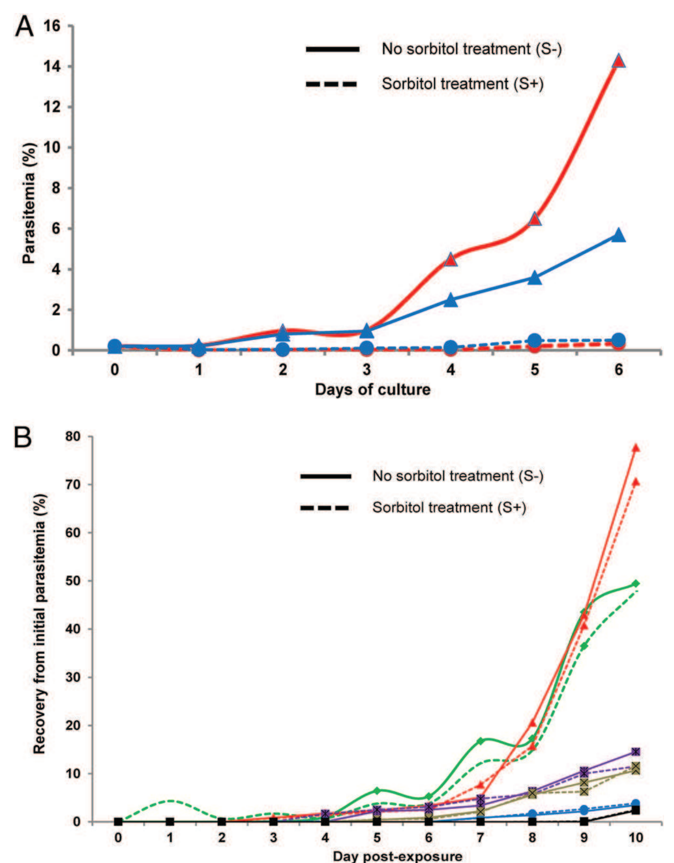

FIG 5 Recrudescence following a 24-h exposure to 700 nM DHA of ring stages with or without sorbitol treatment after drug exposure. (A) Parasitemia of control cultures (no drug exposure) of PL4971 (red) and RT4974 (blue). Cultures received a sorbitol treatment at H24 (S+) (dotted line) or were mock treated (S−) (solid line). Sorbitol treatment induced, as predicted, massive lysis of infected red cells and a dramatic drop of parasite counts (at D6, PL4971 = 14.3% in S− versus 0.2% in S+ and RT4974 = 5.6% in S− versus 0.3% in S+). (B) Recovery of the initial parasitemia of DHA-exposed PL4971 (red), PL5145 (green), PL4992 (purple), RT3592 (brown), RT4974 (blue), and RT5150 (black). Cultures received a sorbitol treatment immediately after a 24-h exposure to 700 nM DHA (S+) (dotted line) or were mock treated (S−) (solid line). In DHA-treated cultures, sorbitol treatment had no impact on the growth curve of cultures (at D10, PL4971 = 77.7% in S− versus 70.6% in S+, PL5145 = 47.8% in S− versus 49.5% in S+, PL4992 = 14.5% in S− versus 11.5% in S+, RT3592= 10.6% in S− versus 11.6% in S+, RT4974 = 3.5% in S− versus 3.9% in S+, and RT5150 = 2.5% in S− versus 2.3% in S+).

age of viable parasites in RSA and a quicker recovery of initial parasitemia after drug removal (Fig. 4).

The most important information gathered from this study is that ring stages from Pailin showed an altered *in vitro* phenotype. They were able to survive a high-dose DHA exposure, unlike the mature stages, which were fully susceptible to DHA. It thus appears that the artemisinin resistance reported in Pailin is associated with an increased number of rings capable of entering a transient developmental arrest and resuming growth after drug removal (Fig. 5B). The *in vitro* phenotype consistently observed in all Pailin lines exposed to DHA thus differs from the usual drug resistance phenotype, in which essentially all parasites survive exposure and proceed unimpaired to schizogony. This is partially consistent with the observations made by Teuscher et al. using artelinic acid-resistant parasite lines where higher proportions of viable rings were observed in the resistant lines after treatment than in sensitive lines (17) and differs from the phenotype of the DHA-resistant laboratory lines derived from the multidrug-resistant Dd2 clone of W2 (18), which multiply in the presence of artemisinins and as a consequence display a higher IC_{50} for DHA than their sensitive parents. In both Pailin and Ratanakiri, DHA seems active in its effect on mature stages but has a reduced efficacy for ring stages, consistent with recent mathematical modeling (13).

Our observation that mature stages of artemisinin-resistant parasites from Pailin remain fully susceptible to DHA toxicity explains why these parasites score as susceptible in the standard *in vitro* test, which monitors DNA synthesis and maturation. We previously reported that parasites from western Cambodia (including Pailin) tended to show 2-to-3-fold-higher geometric mean IC_{50}s for artesunate than parasites from eastern Cambodia (including Ratanakiri) (19). These observations derived from analysis of 820 parasite isolates (495 from western Cambodia and 325 from eastern Cambodia) collected from 2001 to 2007. When stratified by year, however, the geographic difference was signifi-

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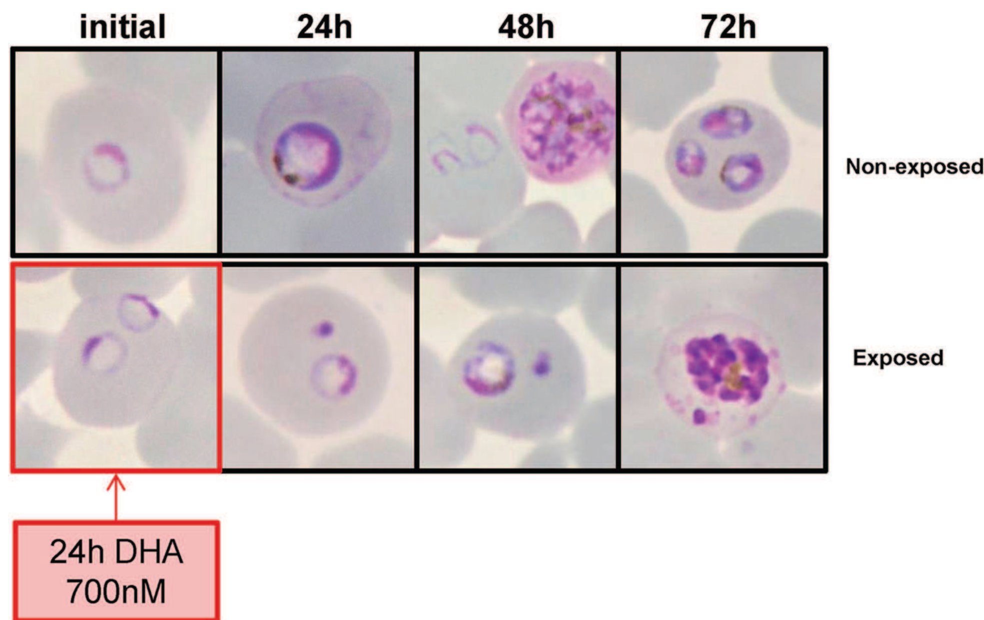

FIG 6 Morphology of PL5145 parasites from H0 to H72 in nonexposed cultures and exposed cultures (24-h exposure to 700 nM DHA). The figure shows Giemsa-stained smears (100× magnification).

cant only for the years 2003 to 2006. In 2007, the most recent year investigated, we did not observe any difference in the artesunate IC_{50}s between western and eastern Cambodia (IC_{50} geometric mean [$GMIC_{50}$] = 1.7 nM [range, 0.4 to 6.7 nM] and $GMIC_{50}$ = 1.4 nM [range, 0.5 to 5.6 nM], respectively). This was confirmed by studies performed in Pailin and Ratanakiri from 2009 to 2011 (9). The artesunate and DHA IC_{50} medians for *P. falciparum* isolates from Pailin and Ratanakiri were not significantly different. Although the data observed here are not fully comparable, as they were obtained with a smaller sample size and with culture-adapted lines, the trend for similar IC_{50}s for artesunate and DHA in Pailin and Ratanakiri in the recent years is in line with the data gathered in 2007 (19) and in 2009 to 2011 (9).

Use of a 24-h drug exposure to 700 nM instead of a 6-h course allowed us to convincingly document cell growth arrest, evidenced by essentially unaltered morphology of ring stages and resistance to sorbitol-induced lysis. The developmental block of DHA-treated ring stages observed here has been reported for numerous lines, including susceptible lines (14, 16, 17), but higher numbers of surviving ring stages were recorded for the artemisinin-resistant F32-ART clone (14) and Dd2-derived artemisinin-resistant clones (18). Here, a substantial fraction of rings from Pailin (3.9% to 20%) were quiescent (i.e., apparently arrested in their development) upon exposure to DHA, contrasting with the small fraction of parasites in reference laboratory lines (W2, HB3, 3D7, 7G8, and G15) (0.6% to 5.4%) and even smaller fraction in parasite lines from Ratanakiri (0.2% to 3.3%). These findings indicate that quiescence is an inherent phenotypic trait of *P. falciparum* parasites and that reduced susceptibility to artemisinin derivatives is associated with a higher proportion of ring stages entering developmental arrest upon exposure to the drug and subsequently exiting quiescence. This seems to account for the observed fundamental difference between the Pailin and Ratanakiri parasite populations, consistent with the evidence of artemisinin resistance being a heritable parasite-encoded trait (11).

After a 6-h exposure to 700 nM DHA and after a 24-h exposure to the same high dose, the quiescent, seemingly growth-arrested parasites were readily detected, as they retained typical ring morphology. In this regard, the phenotype of quiescent parasites observed here and by Witkowski et al. (14) differs from the phenotype of dormant parasites reported by Teuscher et al. (16) and Tucker et al. (15), who describe dormant parasites as having a smaller cytoplasm and more-condensed chromatin, i.e., resembling pyknotic forms more than ring forms.

Our results indicate that after a single dose of 700 nM DHA, all 10 lines from Pailin had a higher proportion of surviving rings than all 10 lines from Ratanakiri or reference laboratory clones. The counts of parasites that resumed growth upon drug removal matched the counts of the surviving ring forms, consistent with the interpretation that these forms were quiescent. This demonstrated that survival of ring-stage parasites from Pailin—and therefore their *in vitro* phenotype of reduced susceptibility to DHA—was mediated by cell growth arrest or by a physiological state of very slow, marginal progression in the cell cycle. This conclusion is substantiated by the positive correlation found between the RSA and the recovery assay at D6 and D10 after a 24-h DHA exposure. We therefore propose that Pailin parasites withstand DHA toxicity thanks to an increased number of rings able to enter quiescence and remain alive at high DHA concentrations. We think that this increased number of quiescent forms accounts for the ability to recover earlier than the susceptible strains and for the ability to recrudesce at higher numbers than susceptible parasites. Quicker recovery and a higher number of recrudescing parasites have been described in artelinic acid-resistant lines (17) and artemisinin-resistant lines (15), although they do not seem attributable to a greater number of dormant rings in these lines.

We have developed here an *in vitro* assay that provides phenotypic partitioning in a clear dichotomy between Pailin and Ratanakiri isolates, the latter exhibiting phenotypes close to those of ART-susceptible laboratory lines. The assay is simple, and it is

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much quicker than the recovery or recrudescence assay. Decline and resumed growth are captured in the RSA, which monitors the number of parasites having survived a short pulse of high-dose DHA. Detection of viable parasites was carried out by microscopy, and only morphologically viable parasites capable of producing a second generation of parasites were numerated, disregarding the parasites with “crisis” or “pyknotic” morphology (Fig. 6; see also Fig. S2 in the supplemental material). Control cultures (nonexposed to DHA) were processed under the same conditions to control for variations due to the fitness or reinvasion capacity of each parasite line. We used here microscopic examination in order to visually inspect and follow events occurring in DHA-treated cultures, including vacuolization and pyknosis or, alternatively, apparent unchanged morphology and subsequent development. Although microscopy remains cheap and reliable, the method is time-consuming and not adapted for high-throughput formats needed for large-scale epidemiological studies. Alternative methods such as fluorescence-activated cell sorter (FACS) analysis using specific viability markers (14, 18, 28) have to be developed or adapted for use with ring stages under field-based conditions. Despite their current limitations, RSA and RA formats provided *in vitro* evidence for the reduced susceptibility to artemisinin in *P. falciparum* ring-stage parasites from western Cambodia so far documented only *in vivo* studies and substantiated by parasite population genetic evidence (4, 11). These phenotypes should help analysis of the underlying genetic traits (12). Work is now warranted to investigate these *in vitro* phenotypes on a larger scale to understand their relationships with *in vivo* clearance and recrudescence data.

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B.W. contributed to the study design, performed experiments and data analysis, and wrote the paper. P.C., S. Ke, and N. Kloeung performed the in vitro assays. N. Khim and S.C. performed genetic polymorphism analyses. O.G. performed FACS analysis. S. Kim, S.D., R.L., P.R., A.M.D., and R.T. coordinated and supervised the clinical studies and provided *P. falciparum* isolates. F.B.-V., A.B., F.A., and J.-C.B. contributed to the study design. O.M.-P. contributed to the study design, the analysis of the data, and the writing of the manuscript. D.M. contributed to the study design and the writing of the manuscript and performed quality control of the data and data analysis. P.R. is a staff member of the World Health Organization.

We alone are responsible for the views expressed in this publication, and they do not necessarily represent the decisions, policy, or views of the World Health Organization.

We declare that we have no conflicts of interest.

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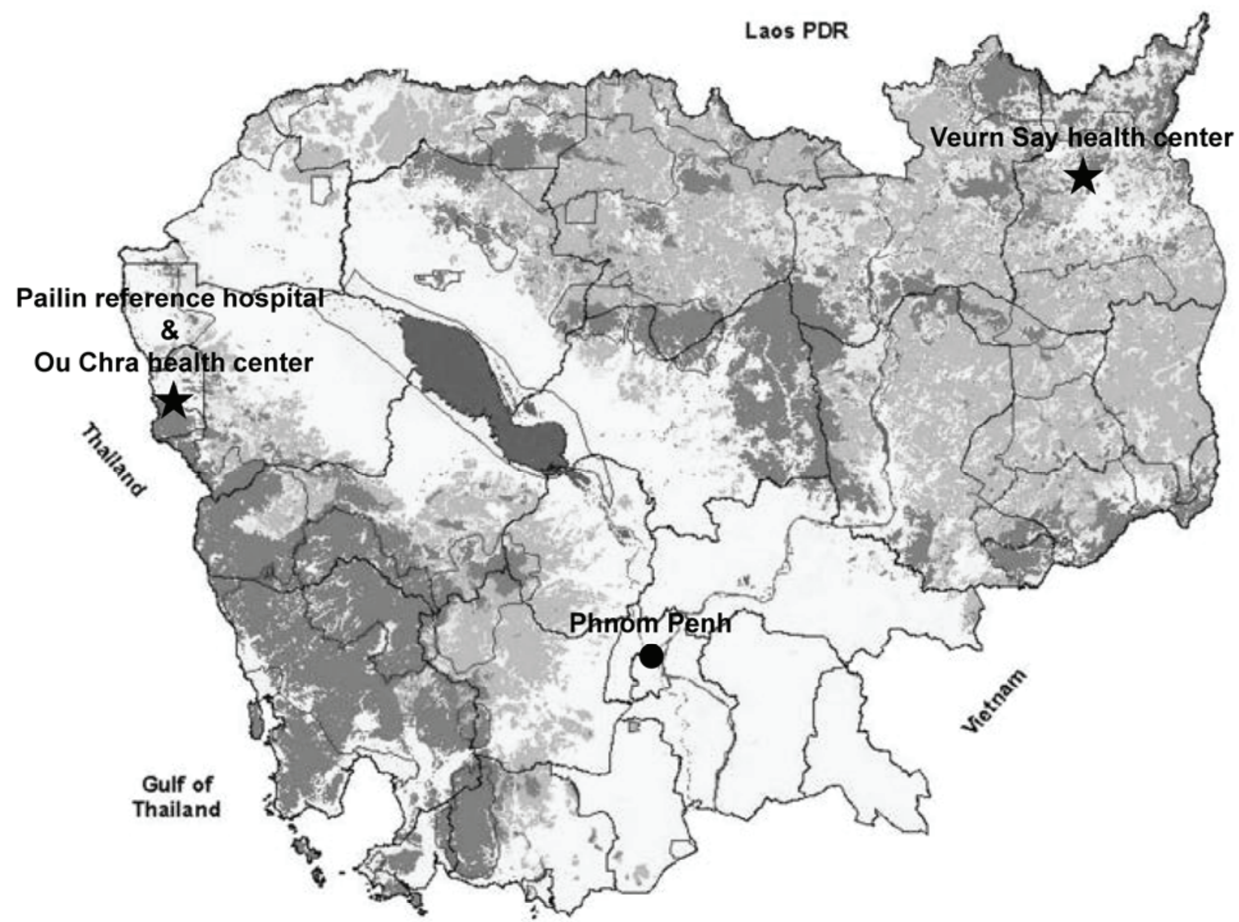
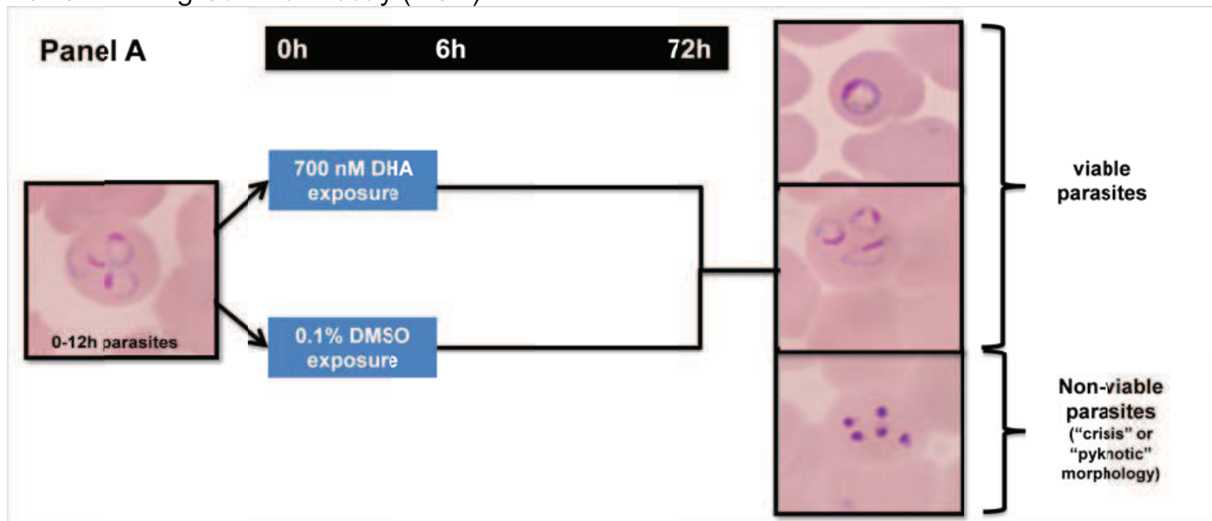
Figure S1. *P. falciparum* isolates collection sites, Cambodia, 2010-2011.

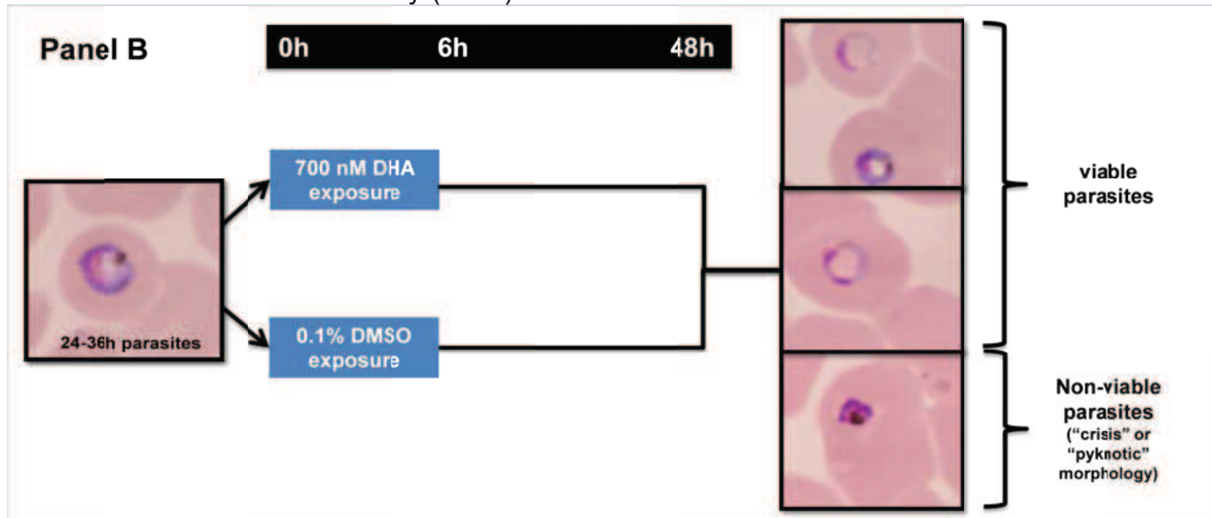


P. falciparum isolates were collected from malaria infected patients in Pailin (artemisinin-resistant area in the western Cambodia) and Ratanakiri (artemisinin-susceptible area in the Eastern Cambodia). Blood samples collected in ACD tubes from patients enrolled in the antimalarial drug resistance surveillance network (conducted by CNM and Institut Pasteur in Cambodia), in TRAC project (conducted by Mahidol-Oxford Tropical Medicine Research Unit) and therapeutic efficacy studies (conducted by CNM and WHO) were transported to Phnom Penh at 4°C within 24-48 hours.

Figure S2. Detailed protocols used to explore parasite stages susceptibility to DHA.
Panel A: Ring Survival Assay (RSA)



Panel B: Mature Survival Assay (MSA).



Synchronized parasites by 5%-sorbitol treatments were exposed 6 hours to 700 nM DHA (~2% parasitemia, 2% hematocrit, 2 ml final volume). Exposed parasites were then washed and resuspended in culture medium. Non-exposed parasites used as controls were exposed 6 hours to 0.1% DMSO and processed in the same conditions. Susceptibility to DHA was assessed microscopically (thin films) by estimating the percentage of viable parasites that had developed into second generation of rings or trophozoites, 66-hours or 42-hours following exposure of ring-stages or mature-stages, respectively. Each smear was read by two independent microscopists (50 consecutive x100 field, ~250 red blood cells per field) and results were expressed as the ratio of the parasite density found in exposed and non-exposed cultures.

Table S1. Ring survival assay (RSA) data of parasites from Pailin, Ratanakiri and reference clones (W2, 3D7, HB3, G15 and 7G8).

ID IPC	No. experiment	Site	66 hours post-exposure (6 hours - 700 nM DHA) reading of								RSA				Growth control
			exposed ring-stages				non-exposed ring-stages								
			M1*	M2**	Mean	SD	M1*	M2**	Mean	SD	Data	Average	SD	RSD	
5145	1	Pailin	2.02	2.03	2.02	0.007	11.23	14.9	13.06	2.59	15.4	14.2	1.4	9.9	3.4
	2	Pailin	0.7	0.68	0.69	0.01	5.3	5.56	5.43	0.18	12.7				2.1
	3	Pailin	1.96	1.22	1.59	0.52	11.0	11.0	11.0	0.0	14.4				1.5
4970	1	Pailin	0.5	0.35	0.42	0.10	2.76	2.21	2.48	0.38	17.1	15.5	1.5	9.8	1.2
	2	Pailin	0.37	0.42	0.39	0.03	2.55	2.6	2.57	0.03	15.4				2.2
	3	Pailin	0.25	0.4	0.32	0.10	2.5	2.12	2.31	0.26	14.0				1.9
4992	1	Pailin	1.02	1.21	1.11	0.13	6.66	8.5	7.58	1.30	14.7	13.8	1.4	10.5	4.3
	2	Pailin	0.36	0.41	0.38	0.03	2.56	2.71	2.63	0.11	14.6				2.7
	3	Pailin	0.78	0.82	0.80	0.03	7.0	6.2	6.6	0.56	12.1				3.0
4248	1	Pailin	0.48	0.67	0.57	0.13	7.9	7.1	7.5	0.56	7.6	7.0	1.0	14.3	3.2
	2	Pailin	0.81	0.65	0.73	0.11	10.4	8.6	9.5	1.27	7.6				3.8
	3	Pailin	0.35	0.33	0.34	0.01	6.4	5.1	5.75	0.91	5.9				3.4
5035	1	Pailin	0.11	0.18	0.14	0.04	2.13	2.17	2.15	0.02	6.7	7.0	1.2	18.0	2.5
	2	Pailin	0.29	0.27	0.28	0.01	3.52	3.15	3.33	0.26	8.4				2.2
	3	Pailin	0.17	0.22	0.19	0.03	3.5	3.1	3.3	0.28	5.9				1.9
5100	1	Pailin	0.05	0.03	0.04	0.01	1.14	1.02	1.08	0.08	3.6	4.2	0.9	21.6	1.7
	2	Pailin	0.12	0.17	0.14	0.03	2.79	2.8	2.79	0.007	5.2				1.9
	3	Pailin	0.12	0.08	0.1	0.03	2.8	2.55	2.67	0.17	3.7				1.7
5160	1	Pailin	0.42	0.3	0.36	0.08	3.16	2.96	3.06	0.14	11.7	19.6	6.8	34.8	1.4
	2	Pailin	0.75	0.7	0.72	0.03	3.23	3.05	3.14	0.12	23.0				1.6
	3	Pailin	0.87	0.77	0.82	0.07	3.6	3.2	3.4	0.28	24.1				2.4
5168	1	Pailin	0.58	0.58	0.58	0.0	4.0	3.57	3.78	0.30	15.3	14.1	4.4	31.0	3.5
	2	Pailin	0.48	0.57	0.52	0.06	2.81	3.08	2.94	0.19	17.9				1.8
	3	Pailin	0.23	0.23	0.23	0.0	2.53	2.4	2.46	0.09	9.3				1.9
5208	1	Pailin	0.12	0.14	0.13	0.008	3.5	3.35	3.425	0.10	3.9	3.9	-	-	1.5
4971	1	Pailin	0.73	0.69	0.71	0.03	4.0	3.85	3.92	0.10	18.1	17.4	2.9	17.1	1.9
	2	Pailin	0.5	0.7	0.6	0.14	4.34	4.15	4.24	0.13	14.1				1.9
	3	Pailin	1.0	1.3	1.15	0.21	5.42	6.1	5.76	0.48	19.9				2.5
5188	1	Ratanakiri	0.005	0.24	0.12	0.16	4.1	3.55	3.82	0.38	3.20	3.3	0.9	27.7	2.6
	2	Ratanakiri	0.04	0.21	0.12	0.12	4.1	5.87	4.98	1.25	2.49				1.5
	3	Ratanakiri	0.06	0.24	0.15	0.12	3.45	3.55	3.50	0.06	4.33				1.8
3592	1	Ratanakiri	0.07	0.12	0.10	0.03	4.42	5.25	4.83	0.58	2.1	1.6	0.4	23.1	1.4
	2	Ratanakiri	0.04	0.06	0.04	0.01	3.5	3.85	3.67	0.24	1.3				3.5
	3	Ratanakiri	0.08	0.06	0.07	0.01	5.36	4.8	5.08	0.39	1.4				2.8
	4	Ratanakiri	0.1	0.17	0.13	0.05	9.0	8.5	8.75	0.35	1.6				2.4
5150	1	Ratanakiri	0.03	0.05	0.04	0.01	4.05	3.2	3.62	0.6	1.1	0.7	0.3	42.4	1.8
	2	Ratanakiri	0.008	0.03	0.02	0.01	4.26	3.9	4.08	0.25	0.5				2.1
	3	Ratanakiri	0.01	0.02	0.015	0.007	2.45	2.2	2.325	0.17	0.6				1.3
5055	1	Ratanakiri	0.005	0.008	0.006	0.002	3.72	4.18	3.95	0.32	0.2	0.2	-	-	1.4
4974	1	Ratanakiri	0.02	0.03	0.02	0.009	2.54	2.64	2.59	0.07	0.9	1.3	0.3	27.2	2.0
	2	Ratanakiri	0.04	0.05	0.04	0.007	4.18	4.03	4.10	0.10	1.1				1.9
	3	Ratanakiri	0.01	0.1	0.05	0.06	3.9	3.2	3.55	0.49	1.6				2.0
	4	Ratanakiri	0.02	0.1	0.06	0.05	4.0	3.9	3.95	0.07	1.6				1.8
5152	1	Ratanakiri	0.02	0.03	0.02	0.008	4.0	3.56	3.78	0.31	0.6	0.6	-	-	2.8
5159	1	Ratanakiri	0.02	0.01	0.01	0.007	2.9	2.4	2.65	0.35	0.6	0.6	-	-	1.5
4880	1	Ratanakiri	0.02	0.05	0.03	0.02	2.28	2.89	2.58	0.43	1.3	1.3	-	-	2.2
5207	1	Ratanakiri	0.02	0.03	0.02	0.009	1.95	2.4	2.17	0.31	1.1	1.1	-	-	2.0
4914	1	Ratanakiri	0.02	0.1	0.06	0.05	2.38	2.25	2.31	0.09	2.5	2.5	-	-	1.8
3D7	1	Reference clone	0.009	0.03	0.01	0.01	2.38	2.25	2.31	0.09	0.8	0.8	-	-	1.9
G15	1	Reference clone	0.1	0.15	0.12	0.03	3.6	3.5	3.55	0.07	3.5	3.5	-	-	1.7
W2	1	Reference clone	0.82	1.05	0.93	0.16	16.4	18.3	17.35	1.34	5.3	5.3	-	-	4.9
7G8	1	Reference clone	0.08	0.06	0.07	0.01	3.92	4.12	4.02	0.14	1.7	1.7	-	-	3.4
HB3	1	Reference clone	0.03	0.01	0.02	0.01	3.2	3.0	3.1	0.14	0.6	0.6	-	-	2.8

*M1: Microscopist 1; **M2: Microscopist 2; SD: Standard deviation; RSD: Relative Standard deviation; Growth control (=parasite density in non-exposed culture/initial parasite density).

Parasite densities at H0 ranged from 0.8% to 4.7% (mean: 1.9%, 95%CI: 1.7%-2.2%) and were not significantly different between both sites (Pailin, mean: 1.7%, range: 0.8%-2.9%, 95%CI: 1.4%-2.0% and Ratanakiri, mean: 1.9%, range: 1.1%-3.7%, 95%CI: 1.5%-2.4%).

Article 6

Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: in-vitro and ex-vivo drug-response studies

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Summary

Background Artemisinin resistance in *Plasmodium falciparum* lengthens parasite clearance half-life during artemisinin monotherapy or artemisinin-based combination therapy. Absence of in-vitro and ex-vivo correlates of artemisinin resistance hinders study of this phenotype. We aimed to assess whether an in-vitro ring-stage survival assay (RSA) can identify culture-adapted *P falciparum* isolates from patients with slow-clearing or fast-clearing infections, to investigate the stage-dependent susceptibility of parasites to dihydroartemisinin in the in-vitro RSA, and to assess whether an ex-vivo RSA can identify artemisinin-resistant *P falciparum* infections.

Methods We culture-adapted parasites from patients with long and short parasite clearance half-lives from a study done in Pursat, Cambodia, in 2010 (registered with ClinicalTrials.gov, number NCT00341003) and used novel in-vitro survival assays to explore the stage-dependent susceptibility of slow-clearing and fast-clearing parasites to dihydroartemisinin. In 2012, we implemented the RSA in prospective parasite clearance studies in Pursat, Preah Vihear, and Ratanakiri, Cambodia (NCT01736319), to measure the ex-vivo responses of parasites from patients with malaria. Continuous variables were compared with the Mann-Whitney *U* test. Correlations were analysed with the Spearman correlation test.

Findings In-vitro survival rates of culture-adapted parasites from 13 slow-clearing and 13 fast-clearing infections differed significantly when assays were done on 0–3 h ring-stage parasites (10·88% *vs* 0·23%; p=0·007). Ex-vivo survival rates significantly correlated with in-vivo parasite clearance half-lives (n=30, *r*=0·74, 95% CI 0·50–0·87; p<0·0001).

Interpretation The in-vitro RSA of 0–3 h ring-stage parasites provides a platform for the molecular characterisation of artemisinin resistance. The ex-vivo RSA can be easily implemented where surveillance for artemisinin resistance is needed.

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Introduction

After the WHO's recommendation[1] to use artemisinin-based combination therapies (ACTs) for the treatment of *Plasmodium falciparum* malaria, the burden of this disease declined substantially.[2] As with earlier antimalarial drugs,[3] parasite resistance to artemisinin and its derivatives has emerged in southeast Asia. Since the first reports in 2008 from Battambang[4] province and 2009 from Pailin[5] province, both in western Cambodia, artemisinin-resistant *P falciparum* malaria has been reported elsewhere in western Cambodia,[6,7] western Thailand,[8] southern Burma,[9] and southern Vietnam.[10] Artemisinin resistance threatens malaria control, treatment, and elimination efforts worldwide.[11,12] To prevent the spread of artemisinin-resistant parasites throughout southeast Asia and to Africa, rapid detection of new artemisinin resistance foci and implementation of containment interventions are a top priority.[13]

Although artemisinin resistance has not been precisely defined, it is recognised as a relatively slow parasite clearance rate in patients receiving an artemisinin or ACT.[14] The parasite clearance half-life can be estimated from frequent parasite density counts in patients with initial parasite densities of 10 000 parasites per µL of blood or greater (ie, ≥0·2% parasitaemia).[15] In regions of low malaria transmission, like Cambodia, parasite clearance studies require screening of thousands of febrile individuals over entire transmission seasons to enrol the few patients (<5%) who meet inclusion criteria and agree to several days of hospitalisation. Such studies are thus logistically and financially demanding, as well as inconvenient for patients and their families. There is therefore an urgent need to develop in-vitro and ex-vivo assay readouts that correlate with parasite clearance half-life.

In-vitro readouts (ie, those obtained from culture-adapted parasite lines in the laboratory) might be useful in elucidating the molecular basis of artemisinin resistance by providing robust phenotypes for genome-wide association studies or the experimental validation of

candidate molecular markers. Ex-vivo readouts (ie, those obtained from uncultured parasite isolates collected directly from patients in the field) might be useful in mapping the geographical spread or worsening of artemisinin resistance in real-time, thus providing actionable information for national malaria control programmes. So far, consistent and significant correlations between half-lives and readouts from any in-vitro or ex-vivo artemisinin susceptibility assay (eg, elevated IC_{50} value—the drug concentration that inhibits parasite growth by 50%) have not been shown.[4–6] One potential reason for this observation is that parasites in these assays are exposed to very low concentrations of dihydroartemisinin (the active metabolite of all artemisinins) for 48–72 h, whereas parasites in vivo are exposed to much higher concentrations of dihydroartemisinin for only 1–2 h.

Artemisinin resistance in drug-selected *P falciparum* lines has been associated with decreased susceptibility of ring-stage parasites[16–18] and, in some lines, mature trophozoite-stage parasites as well.[16,19] Using a novel in-vitro assay (ring-stage survival assay; RSA),[20] we recently measured the susceptibility of 0–12 h post-invasion rings to a pharmacologically relevant exposure (700 nM for 6 h) to dihydroartemisinin. We noted a 17-times higher survival rate of culture-adapted parasite isolates from Pailin province, a region of artemisinin resistance in western Cambodia, compared with those from Ratanakiri province, a region of artemisinin sensitivity in eastern Cambodia. We do not know how this geographical dichotomy relates to the clinical artemisinin resistance phenotype, because ring-stage parasites from patients with known parasite clearance kinetics have not yet been tested in the RSA.

We aimed to assess whether an in-vitro RSA can distinguish culture-adapted *P falciparum* isolates from patients with slow-clearing or fast-clearing infections, to investigate the stage-dependent susceptibility of parasites to dihydroartemisinin in the in-vitro RSA, and to assess whether an ex-vivo RSA can identify artemisinin-resistant *P falciparum* infections in patients with malaria. To mimic the in-vivo exposure of circulating, ring-stage parasites to pharmacologically relevant doses of dihydroartemisinin, we exposed synchronised, ring-stage parasites to brief, high-dose pulses of this drug in the in-vitro RSA. We similarly exposed ring-stage parasites obtained directly from patients in the ex-vivo RSA.

See Online for appendix

Methods

Study design, patients, and drug treatment

We did two clinical studies in Cambodia to measure therapeutic responses to artesunate. One study was done in 2009–10 in Pursat province (western Cambodia; registered with ClinicalTrials.gov, number NCT00341003),[6] where artemisinins have been used for 35 years and artemisinin resistance is well established, and the other in 2012 in Pursat province and also in Preah Vihear (northern Cambodia) and Ratanakiri provinces (eastern Cambodia; registered with ClinicalTrials.gov, number NCT01736319), where ACTs were first used in 2000 and artemisinin resistance has not yet been reported. The studies were done in referral hospitals in each province. The Cambodian National Ethics Committee for Health Research and the US National Institute of Allergy and Infectious Diseases Institutional Review Board approved both studies.

The 2009–10 study in Pursat was previously reported.[6] Patients were treated with oral doses of 4 mg/kg artesunate at 0 h, 24 h, and 48 h, and then 15 mg/kg mefloquine at 72 h and 10 mg/kg mefloquine at 96 h.

In the 2012 study, children older than 1 year and non-pregnant adults with uncomplicated falciparum malaria (parasite density ≥10 000 and ≤200 000 parasites per µL of blood) were enrolled if written informed consent was obtained from patients or parents or guardians of children. Patients with severe malaria, *Plasmodium vivax* infection, haematocrit less than 25%, antimalarial drug use in the past 7 days, or known allergy to artemisinins or piperaquine were excluded. Patients were treated with oral doses of Duo-Cotecxin (containing 40 mg dihydroartemisinin and 320 mg piperaquine per tablet; Holleypharma, China) at 0 h, 24 h, and 48 h. The doses were based on bodyweight: half a tablet (<10 kg), one tablet (10–19 kg), one and a half tablets (20–29 kg), two tablets (30–39 kg), and three tablets (≥40 kg).

Parasite density count, staging, and clearance

In the 2009–10 study, thick blood films were made from samples before the first dose of artesunate (0 h) and then every 6 h until asexual parasitaemia was undetectable.[6] In the 2012 study, blood films were made at 0 h, 2 h, 4 h, 6 h, 8 h, and 12 h, and then every 6 h until parasitaemia was undetectable. Parasite developmental stages at 0 h were estimated as tiny or large rings on the basis of morphological criteria (appendix). After patients completed the study, parasite clearance curves were derived from parasite density counts. The parasite clearance half-life (ie, the time for parasite density to decrease by 50%) was calculated from the slope constant with the parasite clearance estimator.[15,21] The half-life was deemed interpretable when the R^2 value of the slope regression line was greater than 0·8.

In-vitro parasite adaptation

In the 2009–10 study, blood samples were collected into acid-citrate-dextrose vacutainers (Becton-Dickinson, Franklin Lakes, NJ, USA) at 0 h. Parasitised erythrocytes were cryopreserved in Glycerolyte 57 (Baxter Healthcare Corp, Deerfield, IL, USA)[22] immediately or after short-term cultivation, and stored in liquid nitrogen until use.

Isotopic in-vitro sensitivity testing

The in-vitro sensitivity of culture-adapted parasites to artesunate and dihydroartemisinin (obtained from the Worldwide Antimalarial Resistance Network) was assessed with a 48 h isotopic test[20] with drug concentrations ranging from 0·1 nM to 102·4 nM for artesunate, and from

0·0625 nM to 64 nM for dihydroartemisinin. The quality of in-vitro assays was monitored with the *P falciparum* 3D7 line. Results were expressed as the inhibitory concentrations IC_{50} and IC_{90}, defined as the drug concentrations at which 50% or 90% of ^{3}H-hypoxanthine (Amersham, Les Ulis, France) incorporation was inhibited compared with drug-free controls. IC_{50} and IC_{90} values were established by non-linear regression with ICEstimator software.[23,24]

In-vitro survival assays

Culture-adapted parasites were synchronised twice with 5% sorbitol (Sigma-Aldrich, Singapore) at 40 h intervals. Synchronous 10–12 nuclei schizonts were incubated for 15 min at 37°C in RPMI-1640 supplemented with 15 U/mL of sodium heparin (Rotexmedica, Luitre, France) to disrupt agglutinated erythrocytes, purified on a 35%/75% Percoll (Sigma-Aldrich) discontinuous gradient, washed in RPMI-1640, and cultured for 3 h with fresh erythrocytes. Cultures were treated with 5% sorbitol to eliminate remaining schizonts, adjusted to 2% haematocrit and 1% parasitaemia by adding uninfected erythrocytes, and dispensed (2 mL per well in a 24-well culture plate) into two parallel cultures. The $RSA^{0-3\,h}$ was done immediately with 0–3 h postinvasion rings, the $RSA^{9-12\,h}$ with 9–12 h postinvasion rings, and the trophozoite-stage survival assay ($TSA^{18-21\,h}$) with 18–21 h postinvasion trophozoites.

In each assay, parasites were exposed to 700 nM dihydroartemisinin or 0·1% dimethyl sulfoxide for 6 h, washed with 12 mL RPMI-1640 to remove drug, resuspended in complete medium (RPMI-1640, 0·5% Albumax II, 2% heat-inactivated B+ plasma, 50 µg/mL gentamicin), and cultured at 37°C in a tri-gas atmosphere (5% CO_2, 5% O_2, 90% N_2). Thin blood smears were prepared and stained with 10% Giemsa (Merck KGaA, Darmstadt, Germany) for 20 min. Survival rates were assessed microscopically by counting the proportion of viable parasites that developed into second-generation rings or trophozoites with normal morphology at 66 h ($RSA^{0-3\,h}$), 57 h ($RSA^{9-12\,h}$), and 48 h ($TSA^{18-21\,h}$) after drug removal. For each sample, roughly 10 000 erythrocytes were assessed independently by two microscopists (BW and CA) from whom each other's data and half-lives were masked. When the difference between survival rates was greater than 20%, a third microscopist (DM), from whom the data were also masked, assessed the slides.[25] Mean parasite counts were calculated and survival rates expressed as ratios of viable parasitaemias in dihydroartemisinin-exposed and dimethyl sulfoxide-exposed samples.

Ex-vivo survival assay

In the prospective 2012 study, ex-vivo RSAs were done on parasites directly from consecutively enrolled patients in Pursat, Preah Vihear, and Ratanakiri. 2 mL of venous blood were collected into acid-citrate-dextrose vacutainers before the first Duo-Cotecxin dose and processed within 24 h. Plasma was removed and the blood washed three times in RPMI-1640. If the parasitaemia was greater than 1%, it was adjusted to 1% by adding uninfected erythrocytes. Ex-vivo RSAs were done as above except that complete medium did not contain human plasma, parasites were not experimentally synchronised, and three different atmospheres were tested in parallel: tri-gas, 5% CO_2, and candle jar. These atmospheres were used to assess whether ex-vivo RSAs can produce interpretable results in field-based or under-resourced settings where gas cylinders and gas-mixing incubators might not be available or affordable. Smears made 66 h after drug removal were assessed and survival rates calculated as described above. Results were viewed as interpretable if the parasitaemia in the sample exposed to dimethyl sulfoxide was higher than the starting parasitaemia.

For more on the **ICEstimator** see http://www.antimalarial-icestimator.net/index.htm

Parasite genotyping

DNA was extracted from 200 µL of whole blood collected in 2010 at 0 h and from corresponding culture-adapted parasites just before in-vitro assays using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). Parasite genotyping was done as described.[26] 12 single-nucleotide polymorphisms were assessed with a PCR ligase detection reaction fluorescence microspheres assay (appendix).

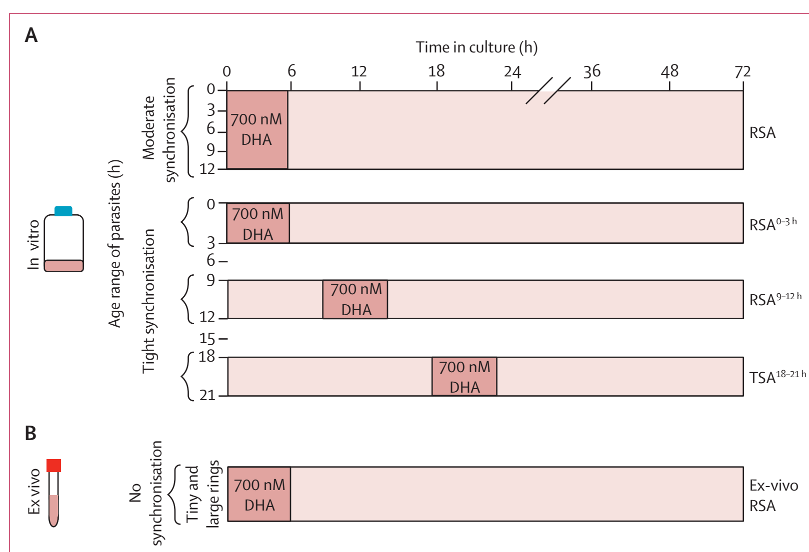

Figure 1: **Dihydroartemisinin survival assays**
Synchronisation and timing of DHA exposure (A) for four in-vitro survival assays—RSA, previously described by Witkowski and colleagues,20 $RSA^{0-3\,h}$, $RSA^{9-12\,h}$, and $TSA^{18-21\,h}$—done on culture-adapted *Plasmodium falciparum* isolates. During their 48 h cycle of intraerythrocytic development, parasites circulate as ring-stages (0–18 h) and then sequester by specifically adhering to the endothelium of microvessels, where they mature into trophozoites (18–36 h) and schizonts (36–48 h). Because of sequestration, clinical studies assess the clearance rate of circulating ring-stage parasites only. In individual patients, the actual age-distribution of parasites circulating in peripheral blood is unknown and can vary from patient to patient. The timing of dihydroartemisinin exposure for the ex-vivo survival assay (B) done on circulating, ring-stage parasites (0–18 h) obtained directly from the blood of patients with uncomplicated malaria. This assay thus measures the dihydroartemisinin susceptibility of the parasite isolate at the same developmental stage and at the same time as the in-vivo parasite clearance study. DHA=dihydroartemisnin. RSA=ring-stage survival assay. TSA=trophozoite-stage survival assay.

Statistical analysis

Data were analysed with Microsoft Excel and MedCalc version 12 (Mariakerke, Belgium). Quantitative data were expressed as median (IQR). Stage-dependent patterns of survival were expressed as the difference between $RSA^{0-3\,h}$ and $TSA^{18-21\,h}$ (Δ). Continuous variables were compared with the Mann-Whitney *U* test. Correlations were analysed with the Spearman correlation test. Ex-vivo RSA values that were obtained in three atmospheric conditions were compared with one-way repeated-measures ANOVA with Bonferroni correction for p values (Friedman test). We deemed significant p values of less than 0·05.

	Fast-clearing parasites (short half-life)	Slow-clearing parasites (long half-life)	p value
$RSA^{0-3\,h}$	0·23 (0·14–2·93, 0·01–51·39)	10·88 (4·75–13·91, 0·16–29·14)	0·007
$RSA^{9-12\,h}$	1·07 (0·77–1·70, 0·06–10·00)	2·12 (1·46–3·55, 0·33–8·00)	0·06
$TSA^{18-21\,h}$	0·99 (0·48–2·20, 0·16–4·10)	1·16 (0·78–2·05, 0·38–5·30)	0·54
Dihydroartemisinin IC_{50}	0·71 (0·58–0·94, 0·29–1·20)	0·79 (0·62–0·11, 0·42–1·51)	0·44
Dihydroartemisinin IC_{90}	2·60 (2·28–3·30, 1·54–4·49)	2·46 (1·78–3·02, 1·48–4·40)	0·36
Artesunate IC_{50}	1·00 (0·84–1·47, 0·28–1·71)	1·11 (0·98–1·84, 0·83–2·50)	0·20
Artesunate IC_{90}	3·32 (2·52–3·94, 2·30–5·80)	3·02 (2·38–3·86, 1·99–6·38)	0·70

Data are median (IQR, range). Percentage survival in $RSA^{0-3\,h}$, $RSA^{9-12\,h}$, and $TSA^{18-21\,h}$, and IC_{50} and IC_{90} values for dihydroartemisinin and artesunate in isotope-based sensitivity assays. p values for significance from Mann-Whitney test. RSA=ring-stage survival assay. TSA=trophozoite-stage survival assay.

***Table:* Parasite survival in in-vitro assays using culture-adapted isolates**

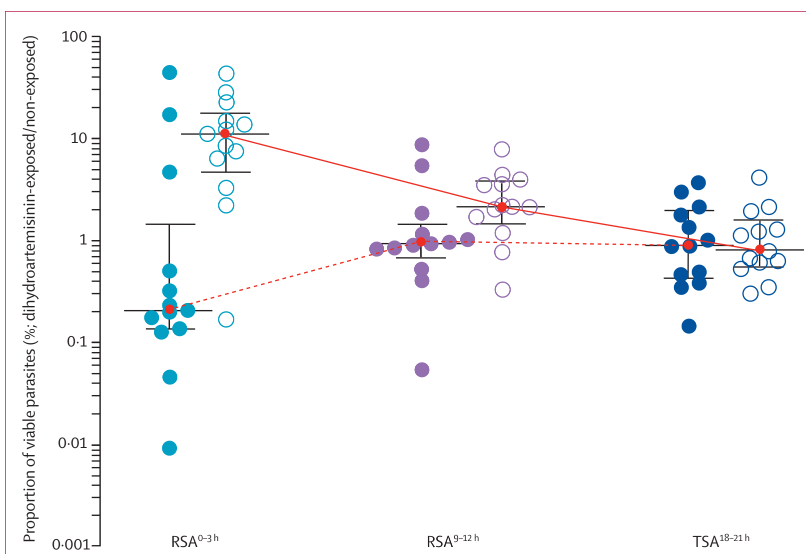

***Figure 2:* In-vitro survival after exposure to dihydroartemisinin**

Results are expressed as the proportion of viable *Plasmodium falciparum* parasites after a 6 h exposure of 0–3 h rings ($RSA^{0-3\,h}$), 9–12 h rings ($RSA^{9-12\,h}$), and 18–21 h trophozoites ($TSA^{18-21\,h}$) to 700 nM dihydroartemisinin compared with dimethyl sulfoxide. These assays were done on culture-adapted parasite isolates obtained from 13 patients with fast-clearing infections (filled circles) and 13 patients with slow-clearing infections (open circles) in Pursat in 2010. The horizontal lines represent the medians and whiskers the IQRs. The solid lines show stage-dependent survival pattern of parasites from slow-clearing infections and the dotted lines the stage-dependent survival pattern of parasites from fast-clearing infections. RSA=ring-stage survival assay. TSA=trophozoite-stage survival assay.

Role of the funding source

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

Results

From the 89 patients enrolled, we selected 18 fast-clearing and 20 slow-clearing parasites representing the lower and upper quartiles of the half-life distribution and adapted them to culture as described.[20] Assays were ultimately done on parasites from 13 fast-clearing and 13 slow-clearing infections; the other 12 selected parasites were excluded from the study because they did not adapt to culture, did not have a corresponding half-life value that was interpretable, or did not show an identical genotype to the parasite originally obtained from the patient (appendix).

Parasites were collected in Pursat in 2010 (appendix), and used in three stage-specific survival assays (figure 1). In the RSA for 0–3 h rings ($RSA^{0-3\,h}$), the median survival rate of slow-clearing parasites was 47 times greater than that of fast-clearing parasites (table, figure 2). By contrast, 9–12 h rings and 18–21 h trophozoites from fast-clearing and slow-clearing infections showed no significant difference in survival (table, figure 2). The stage-dependent survival patterns differed between fast-clearing and slow-clearing parasites (figure 2, appendix). Specifically, the survival rates of slow-clearing parasites decreased with parasite stage, whereas those of fast-clearing parasites increased with parasite stage (Δ=9·9% [IQR 1·7 to 14·4] *vs* –0·3% [–1·1 to 0·4]; p=0·007). In an isotope-based sensitivity assay that monitored replication of parasites exposed to drug for 48 h,[27] fast-clearing and slow-clearing parasites did not differ significantly in IC_{50} and IC_{90} values for artesunate or dihydroartemisinin (table; appendix).

In patients with falciparum malaria, the age distribution of circulating ring-stage parasites is heterogeneous, ranging from 0 h to 18 h at the time of clinical presentation;[28] that is, ring-stage parasites are not necessarily tightly synchronised at the 0–3 h age of development. We therefore sought to assess whether an ex-vivo RSA could distinguish fast-clearing from slow-clearing parasites that have been neither culture-adapted nor experimentally synchronised.

In the prospective 2012 study, 30 (83%) of 36 patients had interpretable half-life values and tri-gas survival rates (appendix), which correlated significantly (figure 3). Parasite survival rates did not differ between the three atmospheres (n=26; p=0·30, Friedman test). The ex-vivo RSA accurately identified artemisinin-resistant infections where they have not been previously described (figure 3, appendix). In Preah Vihear, for example, one parasite with a 12·2% survival rate had an 8·17 h half-life, whereas the other six parasites with a median 0·70% survival rate

(IQR 0·18–2·0) had a median 2·28 h half-life (1·89–3·52). In Ratanakiri, one parasite with a 38·3% survival rate had a 9·06 h half-life, whereas the other ten parasites had a median 0·40% survival rate (0·26–1·48) and a median 2·28 h half-life (1·90–2·64). Our findings suggest that artemisinin-resistant *P falciparum* has spread or independently emerged in northern and eastern Cambodia, a possibility that can now be confirmed with the in-vitro $RSA^{0-3\,h}$.

Discussion

P falciparum isolates from slow-clearing and fast-clearing infections in Cambodia respond differently to a 6 h, 700 nM exposure to dihydroartemisinin. In the $RSA^{0-3\,h}$, rings of slow-clearing parasites had much higher survival rates than those of fast-clearing parasites. In the ex-vivo RSA, survival rates correlated with parasite clearance half-lives. Importantly, the ex-vivo RSA accurately identified slow-clearing infections in Cambodian provinces where they have not yet been described. To our knowledge, these are the first reported in-vitro and ex-vivo dihydroartemisinin susceptibility data that correlate with in-vivo parasite clearance half-lives (panel). These data qualify the in-vitro $RSA^{0-3\,h}$ as a new laboratory test for elucidating the mechanism of artemisinin resistance through molecular studies. These studies might include genome-wide association studies,[29] associating $RSA^{0-3\,h}$ survival rates with whole-genome single-nucleotide polymorphism data, phenotypic screening of parasite progeny clones obtained from genetic crosses between artemisinin-sensitive and artemisinin-resistant parental lines, phenotypic characterisation of the different artemisinin-resistant parasite subpopulations circulating in western Cambodia,[29] and validation of candidate molecular markers through genetic manipulation of parasites.

Our findings also suggest that the ex-vivo RSA is a feasible, convenient method for detecting the spread and emergence of artemisinin resistance in areas where it has not yet been reported (eg, eastern Cambodia), or the worsening of artemisinin resistance where it is entrenched (eg, western Cambodia). Both types of findings from ex-vivo RSAs might inform national malaria control programmes to expand or intensify containment measures. In a screen and confirm approach to support such efforts, we propose that the ex-vivo RSA be used in the field to screen for artemisinin-resistant parasites. Any parasite showing dihydroartemisinin resistance in this assay can then be adapted to short-term culture in a laboratory, genotyped to ensure its identity to the clinical parasite isolate obtained from a patient, and tested to confirm dihydroartemisinin resistance with the in-vitro $RSA^{0-3\,h}$.

For both artemisinin-sensitive and artemisinin-resistant parasites, we show stage-dependent heterogeneity of dihydroartemisinin susceptibility in ring forms. In artemisinin-sensitive parasites, 0–3 h rings were more susceptible to dihydroartemisinin than were

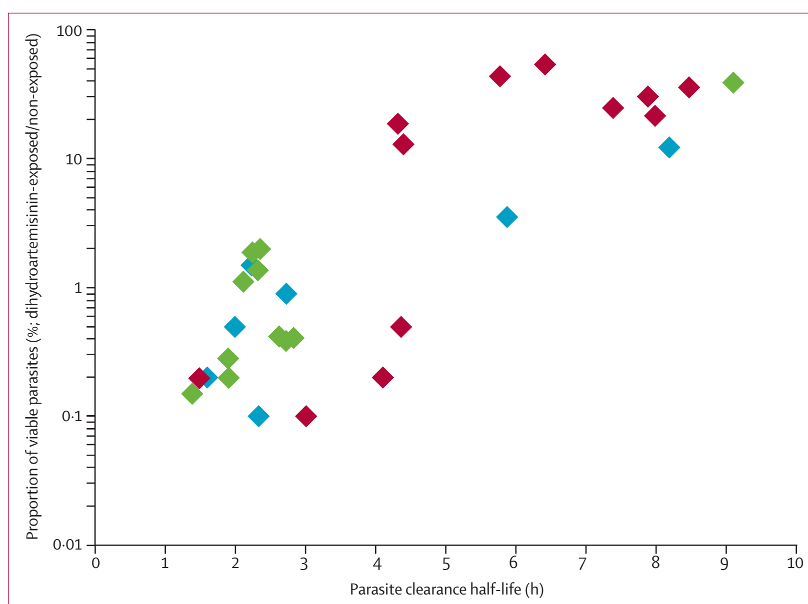

Figure 3: **Correlation of in-vivo parasite clearance half-lives and ex-vivo dihydroartemisinin survival rates**
Ex-vivo ring-stage survival assays (RSAs) were done on parasite isolates obtained directly from patients with malaria in Pursat, Preah Vihear, and Ratanakiri in 2012. Results from the ex-vivo RSAs are expressed as the proportion of viable parasites after a 6 h exposure to 700 nM dihydroartemisinin compared with dimethylsulfoxide-exposed controls. Results from the parasite clearance studies are expressed as the parasite clearance half-life in hours. The proportion of viable parasites in ex-vivo RSAs correlated significantly with the parasite clearance half-life (r=0·74, 95% CI 0·50–0·87; p<0·0001) in Pursat (red), Preah Vihear (blue), and Ratanakiri (green).

9–12 h rings. This finding with clinical parasite isolates is consistent with the recent report that 2–4 h rings of artemisinin-sensitive laboratory lines are specifically hypersensitive to dihydroartemisinin.[18] However, in artemisinin-resistant parasites, 0–3 h rings were less susceptible to dihydroartemisinin than were 9–12 h rings. We tentatively conclude that the susceptibility of Cambodian parasites to dihydroartemisinin is controlled predominantly at the 0–3 h stage of parasite development. This interpretation, and our finding that trophozoites are mostly susceptible to dihydroartemisinin irrespective of half-life, is consistent with mathematical modelling predictions[30] and transcriptomics data[31] from studies of ring-stage parasites.

Half-lives and $RSA^{0-3\,h}$ survival rates were discordant in four patients (figure 2, appendix). Three patients (1007, 1006, and 1009) had fast-clearing infections with parasites showing survival rates of 5·3%, 19·3%, and 51·4%, and a resistant stage-dependent pattern (Δ=1·2%, 17·3%, and 50·2%, respectively). Their patterns differed from those of fast clearing-infections (Δ=–0·7% *vs* 17·3%; p=0·01), being similar to those from slow-clearing-infections (Δ=10·3% *vs* 17·3%; p=0·56; appendix). To explain this discordance, we postulated that these three parasites had already developed into dihydroartemisinin-susceptible, late ring-stage parasites in the patients' blood at the time of the first artesunate dose.

To assess this possibility, we reviewed the initial blood smears from these patients and estimated the relative

Panel: Research in context

Systematic review

We searched PubMed with the terms "artemisinin resistant malaria", limited our search to clinical trials, and used no date or language restrictions. This process produced 56 publications. Any in-vitro or ex-vivo drug assays done in these studies involved the continuous exposure of *Plasmodium falciparum* parasites to very low concentrations of artemisinins during the entire lifecycle of their blood-stage development. Results of these assays have not consistently correlated with clinical efficacy.[4–6] Only four recent publications describe new assays that were specifically designed to measure *P falciparum* susceptibility to artemisinins.[17–20] Klonis and colleagues[18] described an assay working with laboratory-adapted parasites that were artemisinin-sensitive. Witkowski and coworkers[17] and Teuscher and colleagues[19] described assays working with laboratory-adapted parasites and their drug-selected counterparts that became resistant to artemisinins. Witkowski and colleagues[20] described a more relevant study using parasites from western and eastern Cambodia, where parasites are commonly resistant and sensitive to artemisinin respectively. None of these studies were designed to test whether in-vitro susceptibility data correlated with in-vivo efficacy data (ie, parasite clearance rates after artemisinin treatment, the currently accepted clinical phenotype); therefore, these assays could not be clinically validated.

Interpretation

We report for the first time novel in-vitro and ex-vivo ring-stage survival assays (RSAs) that detect artemisinin-resistant, slow-clearing *P falciparum* infections in patients with malaria. In both assays, early ring-stage parasites are exposed to a pharmacologically relevant pulse of dihydroartemisinin and their survival measured 72 h later. With parasites adapted to culture in the laboratory, the in-vitro RSA can be used to discover the molecular mechanisms of artemisinin resistance, to investigate the mode of action of artemisinins, and to identify artemisinin-resistant parasite strains for testing next-generation antimalarial drugs. The ex-vivo RSA with parasites obtained directly from patients with malaria can be easily implemented in field-based settings to monitor the worsening of artemisinin resistance where it is highly prevalent (eg, western Cambodia), and to map its spread or independent emergence elsewhere in the Greater Mekong Subregion. Also, this simple test might readily be established at sentinel sites in sub-Saharan Africa, where the arrival of artemisinin-resistant *P falciparum* is expected to be especially devastating. The ex-vivo RSA can thus provide crucial surveillance data to the national malaria control programmes of all countries threatened by artemisinin-resistant malaria.

For the **standard operating procedures for ex-vivo and in-vitro RSA** see http://www.wwarn.org/toolkit/procedures/invitro

age of their ring-stage parasites just before treatment with artesunate (appendix). In thin blood smears made at 0 h, we noted that these three discordant patients indeed had a two-times lower proportion of tiny rings compared with the 12 concordant patients from the slow-clearing group (ie, those having slow-clearing infections with dihydroartemisinin-resistant parasites; 42·4% *vs* 75·0%; p=0·03). Higher proportions of large, older rings could account for shorter than expected half-lives because these forms are more susceptible to dihydroartemisinin than tiny, young rings. Overall, our findings suggest that the relative abundance of tiny and large rings at the time of the first artemisinin dose affects the parasite clearance half-life, and that accurate ex-vivo staging of parasites is crucial for classifying treatment outcome. In one patient (896), having a slow-clearing infection with a parasite showing a survival rate of 0·2%, and a sensitive stage-dependent pattern (Δ=–1·4%; appendix), we cannot rule out an inadequate immune response to infection[32] or insufficient plasma concentrations of artemisinins.

The $RSA^{0-3\,h}$ survival rate might be crucially informative in ongoing parasite genetics studies[31,33,34] aimed at identifying loci under artemisinin selection, because it is unaffected by in-vivo variables (eg, pharmacokinetics, haemoglobin type, and acquired immunity) that might affect the parasite clearance half-life. Although this phenotype might also be a useful readout in studies to define and validate molecular markers for tracking artemisinin-resistant parasites in the field, the $RSA^{0-3\,h}$ is a laborious assay. By contrast, the ex-vivo RSA saves weeks of effort (results are available in 3 days), and avoids the confounding effects of parasite clone elimination and metabolic changes that might accompany the culture adaptation of parasites. In addition to implementing methods that more precisely establish the age of rings, FACS-based or ELISA-based analysis of parasite viability should improve the throughput of dihydroartemisinin-susceptibility studies. Until such methods are developed and validated, we propose the simple ex-vivo RSA as a highly informative surveillance approach for the identification of artemisinin-resistant parasites in areas where slow parasite clearance is suspected. Investigating the relation between RSA survival rates ex vivo and parasite recrudescence rates in vivo might be useful in assessing the clinical effect of artemisinin resistance.

Contributors

BW, CA, PL, JMA, SK, SD, CMC, WRJT, OM-P, RMF, and DM contributed to study design. NK genotyped parasites. BW, CA, and PC did the in-vitro and ex-vivo drug assays. SSr, SM, CS, BS, and SSu gathered clinical data. BW, CA, OM-P, RMF, and DM analysed data and wrote the report.

Conflicts of interest

We declare that we have no conflicts of interest.

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Supplementary webappendix

This webappendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Witkowski B, Amaratunga C, Khim N, et al. Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: in-vitro and ex-vivo drug-response studies. *Lancet Infect Dis* 2013; published online Sept 11. http://dx.doi.org/10.1016/S1473-3099(13)70252-4.

APPENDIX MATERIAL

Novel phenotypic assays detect artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: in-vitro and ex-vivo drug response studies

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Appendix 3: Patient information and corresponding data from ex-vivo assays performed on *P falciparum* isolates from Pursat, Preah Vihear, and Ratanakiri in 2012.

Appendix 4: Grading of asexual *P falciparum* parasites into two developmental categories: 'tiny' (Panel A) and 'large' (Panel B) rings.

Appendix 5: Selection of *P falciparum* isolates from Pursat in 2010 for culture adaptation and use in in-vitro assays.

Appendix 6: Individual stage-dependent patterns in in-vitro survival assays ($RSA^{0\text{-}3h}$, $RSA^{9\text{-}12h}$, and $TSA^{18\text{-}21h}$) performed on parasite isolates from fast- (Panel A) and slow-clearing (Panel B) infections in Pursat in 2010.

Appendix 1: Patient information and corresponding data from in-vitro assays performed on *P falciparum* isolates from Pursat in 2010.

ID	Age (years)	Sex	Parasitemia at 0 hours (/mm^3)	Parasite clearance half-life (hours)	Fit of parasite clearance curve - R^2	$RSA^{0\text{-}3h}$ survival rate (%)	$RSA^{9\text{-}12h}$ survival rate (%)	$TSA^{18\text{-}21h}$ survival rate (%)	Artesunate IC_{50} (nM)	DHA IC_{50} (nM)	% of tiny rings at 0 hours
906	23	M	33,742	2·20	0.8090	0·15	1·01	0·15	0·28	0·29	42·4
919	37	F	250,000	3·03	0·8847	0·01	0·06	0·17	0·77	0·58	82·9
970	23	M	100,936	3·59	0·8685	0·25	2·1	0·12	0·94	0·88	86·4
189-4	13	M	272,000	3·65	0·9660	0·23	0·46	0·50	1·69	0·97	76·8
915	18	M	50,633	3·69	0·9357	0·35	0·94	0·37	1·34	0·68	65·6
931	29	M	296,666	4·25	0·8884	0·56	1·07	0·52	0·82	0·57	85·4
911	19	M	51,576	4·46	0·8672	0·19	0·60	0·32	0·98	0·76	81·8
918	58	M	351,111	4·54	0·9377	0·14	0·97	0·14	1·52	0·90	77·4
1003	31	M	25,920	4·56	0·9839	0·05	1·16	0·04	1·00	0·60	37·5
1006	**48**	**M**	**11,882**	**4·67**	**0·9680**	**19·32**	**6·27**	**3·08**	**1·33**	**0·71**	**64·8**
1007	**24**	**M**	**16,466**	**4·71**	**0·9237**	**5·30**	**1·30**	**4·08**	**1·71**	**1·20**	**42·4**
1009	**42**	**M**	**27,714**	**4·77**	**0·9314**	**51·39**	**10**	**5·14**	**0·87**	**0·40**	**27·0**
945	10	M	188,500	4·83	0·8714	0·22	1·09	0·20	1·42	1·01	78·1
968	64	M	36,730	7·97	0·9855	8·34	1·71	4·88	0·96	0·68	57·5
818-2	46	M	47,835	7·97	0·9877	13·48	8·00	1·69	1·00	0·81	88·8
976	44	M	65,432	8·21	0·9305	2·18	0·78	2·79	1·14	0·79	65·0
946	17	M	53,626	8·26	0·9458	7·35	1·20	6·13	1·95	1·04	94·4
969	20	M	95,304	8·32	0·9599	6·30	2·12	2·98	2·50	1·51	75·0
950	15	F	79,714	8·54	0·.9495	3·20	3·48	0·92	1·71	1·30	81·8
958	30	M	41,553	8·73	0·9851	29·14	3·62	8·05	1·11	0·71	43·7
896	**21**	**M**	**82,807**	**8·75**	**0·9322**	**0·16**	**0·33**	**0·48**	**0·83**	**0·42**	**76·4**
955	48	M	20,242	9·05	0·9326	11·80	2·20	5·36	1·89	1·18	77·4
938	18	M	22,109	9·11	0·9655	14·33	4·00	3·58	0·85	0·49	57·0
990	31	M	18,125	9·45	0·9775	12·60	3·00	4·20	1·09	0·55	75·0
922	26	M	42,240	9·72	0·9589	21·90	2·10	10·43	1·80	0·95	74·5
956	20	M	48,000	10·08	0·9489	10·88	2·01	5·42	1·11	0·69	84·8

Discordant samples are shown in bold.

Appendix 2: Protocols, PCR/nested PCR primer sequences, and LDR probe sequences used to genotype *P falciparum* isolates obtained in Pursat in 2010.

Assay No.	Outer PCR primer sequences (5'-3')	Inner PCR primer sequences (5'-3')	SNP	Upstream allele-specific probe sequence	Downstream conserved probe sequences (with 5' phosphorylation and 3' biotinylation)
3	TGGAAATACACAATTCAATG	TTCCAAAACTATGTTTGCTGCT	C	cacttaattcattctaaatctatcTTTCAAATGTTATTTTCAACTATGTTAAGTAAC	GATGCAAATAATCTTGATAAAGTATATGG
	CGAATGTTTTTCCATATTTT	TGCAGTGGTACTTGTTGCTACC	T	tactacttctataactcacttaaaTTTCAAATGTTATTTTCAACTATGTTAAGTAAT	
4	CCAACCAACGAACACAAATAC	AGGAAAATGCTCCGGTAACT	T	actacttattctcaaactctaataGAAAAAAATAATTTGAACAATAAAACTTATAATAA	CATGAACGAGTCACCAAATAATATG
	TGGTTGACTGTTATTGGGGTA	GGTTCATATTATTTGGTGACTCG	C	acttatttcttcactactatatcaGAAAAAAATAATTTGAACAATAAAACTTATAATAG	
7	TGAATGTAATATAAATCAGGTTG	CTGAAAAATCGGATGAATGG	G	cactacacatttatcataacaaatAAGGAGATAGTGTTGGGGGC	ATTGCTACATGCATTATACAAAATCC
	GGCTGGAATAGATAAAATCA	GGCTAGCTCAGCTTCCAAT	A	aactttctctctctattcttatttAAGGAGATAGTGTTGGGGGT	
8	CGAATTTAAGTACCTTAGGAAA	TCACAACGTCCATATGTTGAA	G	tcatcactttctttactttacattTGATGAAAGCCACCGAACTC	ATATTTATGGATGAACATTATATTAATAAAGATAT
	TCATAAAGTTTTTATTGTCTTCA	TCATTATCACCTACTTTCTGTACCA	A	tacacaatattcatcataactaacTGATGAAAGCCACCGAACTT	
9	GAGGATGTATACCATTAGCTG	GATGAGTTAGCAACGAAACCA	T	cataatcaatttcaactttctactCCATCATATAAATATTTCTATATTCCATTAGCT	AAATTCTTAGGAAGCTTTTTTCCAAG
	ATCATTCATATGTGGAAACA	AACGTAAACCAGGAGTAAGACG	A	caaatacataatcttacattcactCCATCATATAAATATTTCTATATTCCATTAGCA	
12	ATACACTAAACGCAAAACCT	CATTATGCGAATGCGATCTA	G	ctttctcatactttcaactaatttAATGGAAAATTTTGATGATATTTTATTAAG	TGAAAATGAAAAAGAATTATCTTCATATAAT
	TGTTAATTCCTTTTCGATTT	CGTTTATATTGCAACATTTCTTCA	A	tcaaactctcaattcttacttaatAATGGAAAATTTTGATGATATTTTATTAAA	
13	TGACAAACAAGTATATAATAATAAGAG	TGTTGTTGGTGAATACAATGAAA	G	cttaacatttaacttctataacacAAATAACAATGAACATCATCATGATG	GTTCAGTTATTCCAAATAATTTTTGTAATAA
	TGTTTTAAAAGTCGTGGATA	TCGTACCACCATTAACATTTTG	A	tacaacatctcattaacatatacaAAATAACAATGAACATCATCATGATA	
15	CATAAATAAAACTTTCGCTGA	TGGAATGATTTGAGCAATAGAA	C	ttaaacaatctactattcaatcacAAATTCAAATTATGTTCACAGGAATAAAC	AAAATGATAAGCTTTTTCGTGATGA
	ATTTTCAATATCATCTTCTTTACA	AATACCCATGATATCACATTCCA	A	tctctttaaacacattcaacaataAAATTCAAATTATGTTCACAGGAATAAAA	
16	ATCATCTGTATTTTGTTATTATGA	AATCTTTTCCAGTTATTTTCTATCCA	C	aatcaacacacaataacattcataACCTTCCATATCTAAAAAAACTTCATTC	AAAATCATAGACAAAAAAAAAACAGTTTC
	GTTAGACAATTTTGCTACACTT	CATGGGGGTATGTAATTTGG	A	caatttacatttcactttcttatcACCTTCCATATCTAAAAAAACTTCATTA	
19	TCACAAACAAATAACAATGAA	AAAAGCAATTCCACAAGAACC	A	ttcttcattaacttctaatcttacCCTACATTAAATGAAAATGAAAACGTTA	CTCCCAAACCATCTGAAGGT
	ACATGTTTTGGACCATCTAC	CTGGTGTTTCCTTTTTATTTGG	C	ttaacaacttatacaaacacaaacCCTACATTAAATGAAAATGAAAACGTTC	
20	AATATATCTGTATTTGCTAACATGA	TGTGTTTTATTTTTAGTGTGAGCTTT	C	cataatcaatttcaactttctactCAAAATATCAACAAGAAAAACATAATTACTC	TTGGATGAAATTTCTTGATGAATATAA
	TGTAACAAGGAATGACAAAA	AGAGGATATCCAATAGGGTGCT	T	caaatacataatcttacattcactCAAAATATCAACAAGAAAAACATAATTACTT	
24	CGATTTAATTACTGTTTTGAGA	AACAAATCATCAATTAAGTCATCC	G	cacttaattcattctaaatctatcAATTAGAAAATACACAAAATTATCAAAAAAG	AATTGAAAATTTAAAAAATGTTATTGTTTC
	TTGGTTTACAATTAGTTCTAGC	TGAGGAATAGGTTCATATGCTG	T	tttacaaatctaatcacactatacAATTAGAAAATACACAAAATTATCAAAAAAT	

First-round PCRs were performed in the following reaction mixture: 2·5 µL 10X buffer, 2·5 mM $MgCl_2$, 0·2 mM each deoxynucleoside triphosphate, 0·25 µM each primer, 1·25 U FirePol Taq polymerase (Solis Biodyne, Tartu, Estonia), and 5 µL DNA template. Nested PCRs were performed in the same reaction mixture with 3 µL of first-round PCR products (diluted 1:10) added. PCR amplifications were performed under the following conditions: first-round PCR - 95°C for 15 min and 30 cycles at 95°C for 30 s, 52°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min; nested PCR - 95°C for 15 min and 40 cycles at 95°C for 10 s, 57°C for 15 s, 72°C for 20 s, and a final extension at 72°C for 10 min. As previously described,[1-3] a ligase detection reaction between modified upstream allele-specific (with unique 5' extremity TAG sequences) and downstream conserved sequence primers (with a 5' phosphorylation and 3' biotinylation) were performed using 1 µL of nested PCR products in 15 µL solution of 20 mM Tris-HCl buffer (pH 7·6), 25 mM potassium acetate, 10 mM magnesium acetate, 1 mM NAD+, 10 mM dithiothreitol, 0·1% Triton X-100, 10 nM each LDR probe, and 2 U of Taq

DNA ligase (New England Biolabs, Beverly, MA, USA). Reaction mixtures were heated to 95°C for 1 min, followed by 32 cycles at 95°C for 15 s and 60°C for 2 min. In a second step, 5 µL of multiplex LDR products were added to 60 µL of hybridization solution (3 M tetramethylammonium chloride [TMAC], 50 mM Tris-HCl [pH 8·0], 3 mM EDTA [pH 8·0], 0·10% sodium dodecyl sulfate) containing 2500 MagPlex-TAG Microspheres® (Luminex, Austin, TX, USA) for each allelic set, heated to 95°C for 90 s and incubated at 37°C for 40 min to allow hybridization between SNP-specific LDR products and microsphere-labelled anti-TAG probes. Following hybridization, 6 µL of streptavidin-R-phycoerythrin (Molecular Probes, Eugene, OR, USA) in TMAC hybridization solution (20 ng/µL) was added and incubated at 37°C for 40 min in Costar 6511 M polycarbonate 96-well V-bottom plates (Corning Inc., Corning, NY, USA). Detection of SNP-specific products was performed through a MagPix machine (Luminex). Fluorescence data were managed by xPONENT software (Luminex) and entered into Microsoft Excel software (Microsoft Office 2010). In each run, samples were analyzed with 3D7, Dd2, and HB3 genomic DNA controls and no template control.

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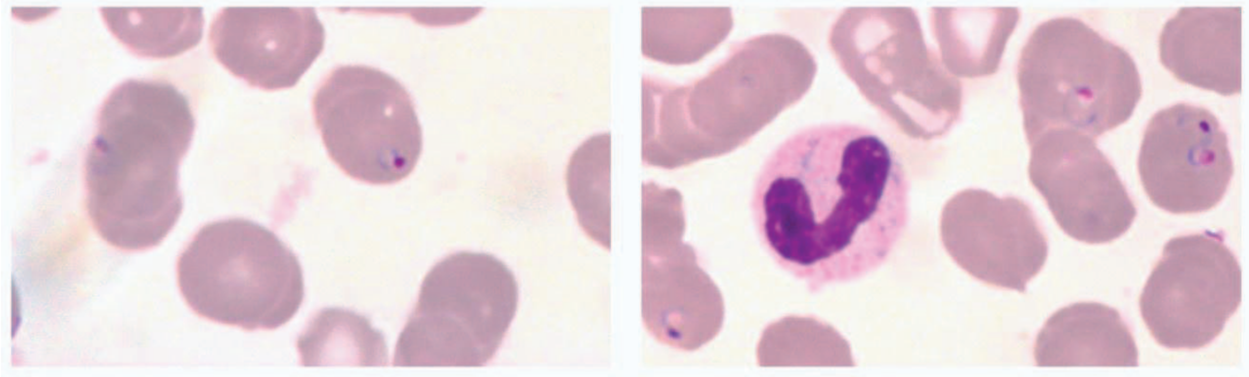
Appendix 3: Patient information and corresponding data from ex-vivo assays performed on *P. falciparum* isolates from Pursat, Preah Vihear, and Ratanakiri in 2012.

ID	Site	Age (year)	Sex	Parasitemia at 0 hours (/mm^3)	Parasite clearance half-life (hours)	Fit of parasite clearance curve - R^2	Ex-vivo RSA value (%)		
							tri-gas	candle-jar	5%CO_2
163-KH1-005	Pursat	26	M	39,412	7·97	0·9628	20·95	11·05	22·83
163-KH1-006	Pursat	18	M	74,847	4·32	0·9899	18·55	22·40	19·05
163-KH1-007	Pursat	17	M	22,638	5·76	0·9883	42·51	39·06	22·.23
163-KH1-013	Pursat	45	M	25,374	7·87	0·9734	29·54	38·25	24·17
163-KH1-015	Pursat	23	M	24,434	4·37	0·9850	12·83	14·85	17·94
163-KH1-016	Pursat	31	M	55,58	4·33	0·9860	0·54	1·12	0·31
163-KH1-018	Pursat	29	M	77,333	6·42	0·9687	54·48	49·01	51·16
163-KH1-021	Pursat	23	M	42,061	3·00	0·9905	0·12	0·01	0·14
163-KH1-022	Pursat	58	F	10,892	7·35	0·9862	24·18	16·29	14·09
163-KH1-027	Pursat	18	M	15,669	4·09	0·9415	0·19	0·06	0·40
163-KH1-030	Pursat	17	F	102,222	1·49	0·9871	0·20	0·06	0·04
163-KH1-031	Pursat	16	F	108,102	8·46	0·9712	35·03	27·89	23·35
163-KH2-005	Preah Vihear	31	M	142,857	2·73	0·9665	0·91	0·20	0·71
163-KH2-009	Preah Vihear	45	F	128	2·23	0·9909	1·50	0·47	ND[1]
163-KH2-010	Preah Vihear	31	M	86,792	8·16	0·9809	12·23	NI[2]	11·98
163-KH2-016	Preah Vihear	59	M	73,379	1·98	0·9172	0·50	0·45	0·18
163-KH2-020	Preah Vihear	24	F	41,859	2·33	0·9726	0·11	0·40	0·26
163-KH2-023	Preah Vihear	40	F	42,772	1·61	0·9917	0·20	0·25	0·13
163-KH2-024	Preah Vihear	35	F	16,236	5·87	0·9405	3·47	1·39	2·60
163-KH3-002	Ratanakiri	25	M	21,587	1·88	0·9862	0·28	ND	ND
163-KH3-004	Ratanakiri	19	M	87,23	2·23	0·9887	1·86	ND	ND
163-KH3-005	Ratanakiri	13	F	31,17	2·73	0·9627	0·38	0·47	0·78
163-KH3-008	Ratanakiri	32	M	10,614	9·06	0·9279	38·59	54·51	36·82
163-KH3-010	Ratanakiri	50	M	53,64	1·36	0·9693	0·14	0·30	0·01
163-KH3-012	Ratanakiri	14	F	12,504	2·83	0·9683	0·40	0·10	0·25
163-KH3-018	Ratanakiri	19	F	31,883	2·34	0·9209	1·98	1·33	1·93
163-KH3-019	Ratanakiri	14	F	37,487	1·89	0·9935	0·20	0·25	0·23
163-KH3-022	Ratanakiri	18	M	30,189	2·61	0·9904	0·41	0·77	0·70
163-KH3-023	Ratanakiri	34	M	56,901	2·10	0·9663	1·09	0·74	1·08
163-KH3-025	Ratanakiri	11	F	49,582	2·32	0·9669	1·35	0·10	0·35

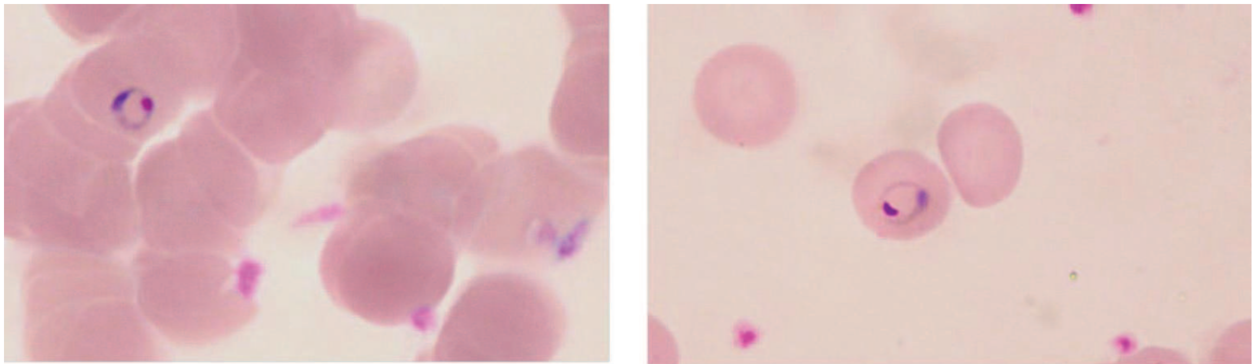
[1]Not done
[2]Not interpretable
KH1, KH2, and KH3 are identifying codes for Pursat, Preah Vihear, and Ratanakiri, respectively; these codes are *not* related to the parasite subpopulations reported by Miotto et al. (*Nat Genet*, 2013).

Appendix 4: Grading of asexual *P falciparum* parasites into two developmental categories: 'tiny' (Panel A) and 'large' (Panel B) rings.

Panel A

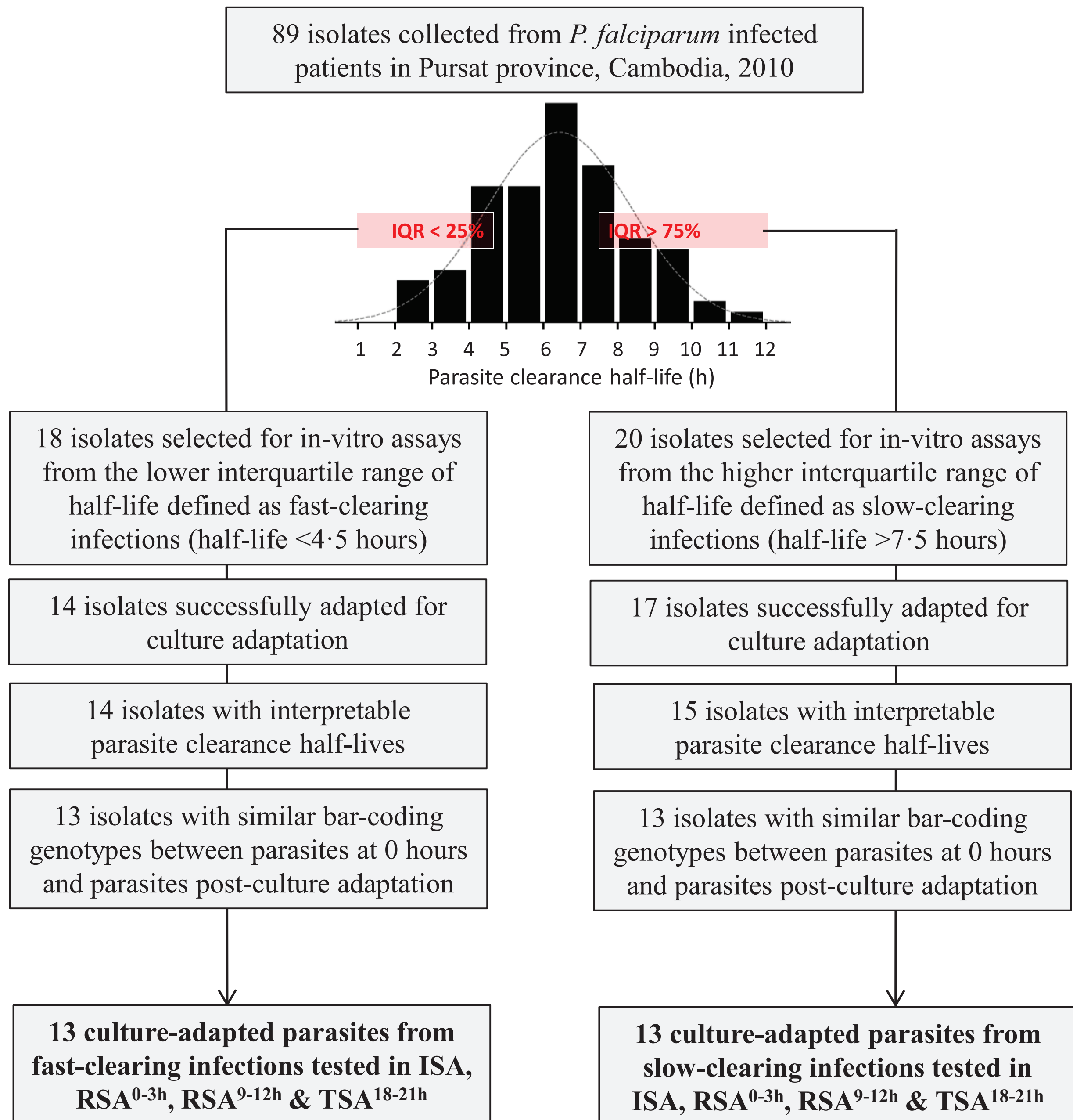


Panel B



Representative photomicrographs of *P. falciparum* isolates collected from patients just prior to receiving a first dose of artesunate. Giemsa-stained thin blood films are shown. Rings were classified as 'tiny rings' when the width of the cytoplasm band was less than, or equal to, half of the diameter of the nucleus (Panel A) and as 'large rings' when the width of the cytoplasm band was greater than the diameter of the nucleus (Panel B).

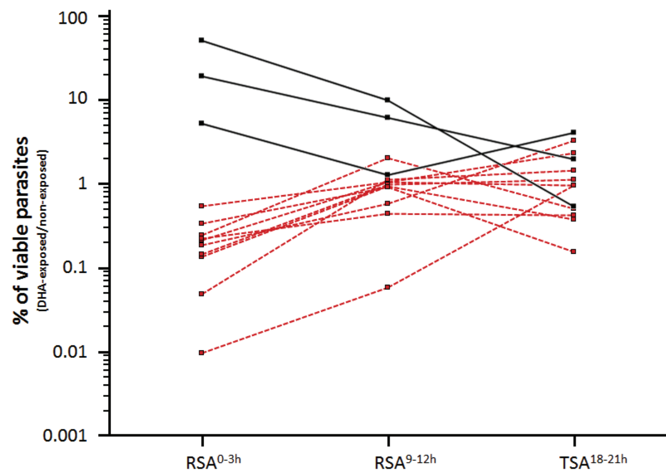
Appendix 5: Selection of *P falciparum* isolates from Pursat 2010 for culture adaptation and use in in-vitro assays.



ISA: Isotope-based assay; RSA^{0-3h}: Ring-stage survival assay with 0-3 hour rings; RSA^{9-12h}: Ring-stage survival assay with 9-12 hour rings & TSA^{18-21h}: Trophozoite-stage survival assay with 18-21 hour trophozoites.

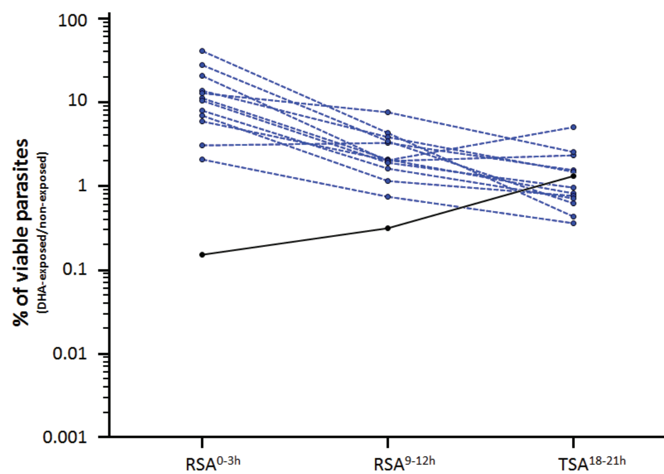
Appendix 6: Individual stage-dependent patterns in in-vitro survival assays (RSA$^{0\text{-}3h}$, RSA$^{9\text{-}12h}$, and TSA$^{18\text{-}21h}$) performed on parasite isolates from fast- (Panel A) and slow-clearing (Panel B) infections in Pursat in 2010.

Panel A



The dotted red lines represent the stage-dependent survival patterns of parasites that show 'concordance' between half-lives and RSA$^{0\text{-}3h}$ survival rates (Δ= -0·7%) and the black solid lines represent the stage-dependent survival patterns of parasites that show 'discordance' between half-lives and RSA$^{0\text{-}3h}$ survival rates (Δ= 17·3%, P=0·01, Mann-Whitney *U* test).

Panel B



The dotted blue lines represent the stage-dependent survival patterns of parasites that show 'concordance' between half-lives and RSA$^{0\text{-}3h}$ survival rates (Δ= 10·3%) and the black solid line shows the stage-dependent survival pattern of the parasite that showed 'discordance' between the half-live and RSA$^{0\text{-}3h}$ survival rate (Δ= -1·2%).

Article 7

ARTICLE

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A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria

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Plasmodium falciparum resistance to artemisinin derivatives in southeast Asia threatens malaria control and elimination activities worldwide. To monitor the spread of artemisinin resistance, a molecular marker is urgently needed. Here, using whole-genome sequencing of an artemisinin-resistant parasite line from Africa and clinical parasite isolates from Cambodia, we associate mutations in the _PF3D7_1343700_ kelch propeller domain ('K13-propeller') with artemisinin resistance _in vitro_ and _in vivo_. Mutant K13-propeller alleles cluster in Cambodian provinces where resistance is prevalent, and the increasing frequency of a dominant mutant K13-propeller allele correlates with the recent spread of resistance in western Cambodia. Strong correlations between the presence of a mutant allele, _in vitro_ parasite survival rates and _in vivo_ parasite clearance rates indicate that K13-propeller mutations are important determinants of artemisinin resistance. K13-propeller polymorphism constitutes a useful molecular marker for large-scale surveillance efforts to contain artemisinin resistance in the Greater Mekong Subregion and prevent its global spread.

The emergence of *Plasmodium falciparum* resistance to artemisinin derivatives (ART) in Cambodia threatens the world's malaria control and elimination efforts[1,2]. The risk of ART-resistant parasites spreading from western Cambodia to the Greater Mekong Subregion and to Africa, as happened previously with chloroquine- and sulphadoxine/pyrimethamine-resistant parasites[3–5], is extremely worrisome. Clinical ART resistance is defined as a reduced parasite clearance rate[1,6–10], expressed as an increased parasite clearance half-life[11,12], or a persistence of microscopically detectable parasites on the third day of artemisinin-based combination therapy (ACT)[2]. The half-life parameter correlates strongly with results from the *in vitro* ring-stage survival assay ($RSA_{0-3\,h}$) and results from the *ex vivo* RSA[13], which measure the survival rate of young ring-stage parasites to a pharmacologically relevant exposure (700 nM for 6 h) to dihydroartemisinin (DHA)—the major metabolite of all ARTs. However, the present lack of a molecular marker hampers focused containment of ART-resistant parasites in areas where they have been documented and hinders rapid detection of these parasites elsewhere, where ACTs remain the most affordable, effective antimalarials. To detect and monitor the spread of ART resistance, a molecular marker for widespread use is needed.

Recent genome-wide analyses of *P. falciparum* isolates have provided evidence of recent positive selection in geographic areas of ART resistance[9,14–16]. Whereas parasite heritability of the clinical phenotype is above 50%, no reliable molecular marker has yet been identified. One possible explanation is that the parasite clearance half-life is not only determined by the intrinsic susceptibility of a parasite isolate to ART, but also by its developmental stage at the time of ART treatment and host-related parameters such as pharmacokinetics and immunity[17]. This issue was recently highlighted in patients presenting discordant data between parasite clearance half-life *in vivo* and $RSA_{0-3\,h}$ survival rate *in vitro*[13]. Moreover, genome-wide association studies (GWAS) are confounded by uncertainties about parasite population structure. Recent evidence for several highly differentiated subpopulations of ART-resistant parasites in western Cambodia[15] suggests that distinct emergence events might be occurring. An alternative strategy to discover a molecular marker is to analyse mutations acquired specifically by laboratory-adapted parasite clones selected to survive high doses of ART *in vitro*, and use this information to guide analysis of polymorphism in clinical parasite isolates from areas where ART resistance is well documented at both temporal and geographical levels. Here we used this strategy to explore the molecular signatures of clinical ART resistance in Cambodia, where this phenotype was first reported[1,8].

A candidate molecular marker of ART resistance

The ART-resistant F32-ART5 parasite line was selected by culturing the ART-sensitive F32-Tanzania clone under a dose-escalating, 125-cycle

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*These authors contributed equally to this work.

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regimen of artemisinin for 5 years[18]. Whole-genome sequences were obtained for both F32-ART5 and F32-TEM (its sibling clone cultured without artemisinin) at 460× and 500× average nucleotide coverage, respectively. Compared to F32-TEM, no deleted genes were identified in F32-ART5. The exomes of F32-ART5 and F32-TEM were compared after excluding (1) genes from highly variable, multi-gene families (*var*, *rifin* and *stevor*), (2) positions with coverage lower than 25% of the mean coverage of the parasite line, (3) single-nucleotide polymorphisms (SNPs) found to be mixed in F32-ART5, given that acquired ART-resistance mutation(s) could be expected to be fixed in the sample after 5 years of continuous pressure, (4) SNPs shared between F32-ART5 and the ART-sensitive 3D7 parasite strain and (5) synonymous SNPs (Extended Data Fig. 1).

This analysis identified eight mutations in seven genes that were subsequently confirmed by Sanger sequencing of PCR products (Extended Data Table 1). Each gene harbours one mutant codon in F32-ART5 compared to F32-TEM, F32-Tanzania or 3D7 (Extended Data Table 2). Information on the expression of the genes and the biological function of the proteins are listed in Extended Data Table 3. Only one of these genes, cysteine protease falcipain 2a (*PF3D7_1115700*), has previously been associated with *in vitro* responses to ART[19]. To determine when each mutation arose in the F32-ART5 lineage, we analysed the whole-genome sequences of parasites at various drug-pressure cycles (Fig. 1). This analysis showed that the *PF3D7_0110400* D56V and *PF3D7_1343700* M476I mutations were acquired first, during the steep increase of ART resistance, and remained stable thereafter. Importantly, the appearance of these two mutations is associated with an increase in the $RSA_{0–3\,h}$ survival rate, from less than 0.01% to 12.8%. Subsequent PCR analysis of the *PF3D7_1343700* locus detected the M476I mutation after 30 drug-pressure cycles, consistent with the sharp increase in $RSA_{0–3\,h}$ survival rate observed thereafter. The other SNPs appeared stepwise at later stages of selection: *PF3D7_0213400* (68 cycles); *PF3D7_1115700* (98 cycles); *PF3D7_1302100*, *PF3D7_1459600* and *PF3D7_1464500* (120 cycles) (Extended Data Table 2). These data indicate that the *PF3D7_1343700* M476I mutation increased the resistance of F32-Tanzania to DHA in the $RSA_{0–3\,h}$.

To explore whether these mutations are associated with ART resistance in Cambodia, we investigated sequence polymorphism in all seven genes by mining whole-genome or Sanger sequences for 49 culture-adapted parasite isolates collected in 2010–2011 (see Methods). We chose these isolates based on their differential $RSA_{0–3\,h}$ survival rates (Supplementary Table 1) and their sequences were compared to those of control parasites lines 3D7, 89F5[20] and K1992 (see Methods). Three genes (*PF3D7_0110400*, *PF3D7_0213400* and *PF3D7_1302100*) encode a wild-type sequence for all parasite isolates. The other four genes show intra-population diversity, with previously reported or novel SNPs (Supplementary Table 1). *PF3D7_1115700* has 11 SNPs that are not associated with $RSA_{0–3\,h}$ survival rates ($P = 0.06$, Kruskal–Wallis test). *PF3D7_1459600* has 6 SNPs that are not associated with survival rates ($P = 0.65$). *PF3D7_1464500* has 12 SNPs previously reported in older isolates from southeast Asia, including the ART-susceptible Dd2 line[21], probably reflecting a geographic signature. These SNPs also show no significant association with survival rates ($P = 0.42$). Therefore, these six genes were not studied further.

In contrast, *PF3D7_1343700* polymorphism shows a significant association with $RSA_{0–3\,h}$ survival rates (Fig. 2). Indeed, $RSA_{0–3\,h}$ survival rates differ substantially between parasite isolates with wild-type (median 0.17%, range 0.06–0.51%, $n = 16$) or mutant (18.8%, 3.8–58%, $n = 33$) K13-propeller alleles ($P < 10^{-4}$, Mann–Whitney U test) (Supplementary Table 1). Four mutant alleles are observed, each harbouring a single non-synonymous SNP within a kelch repeat of the C-terminal K13-propeller domain, namely Y493H, R539T, I543T and C580Y located within repeats no. 2, 3, 3 and 4, respectively. Both the K1992 and the ART-susceptible 89F5 lines carry a wild-type K13-propeller. There are no associations between polymorphisms in the K13-propeller and those in the other candidate genes (Supplementary Table 1). Based on these

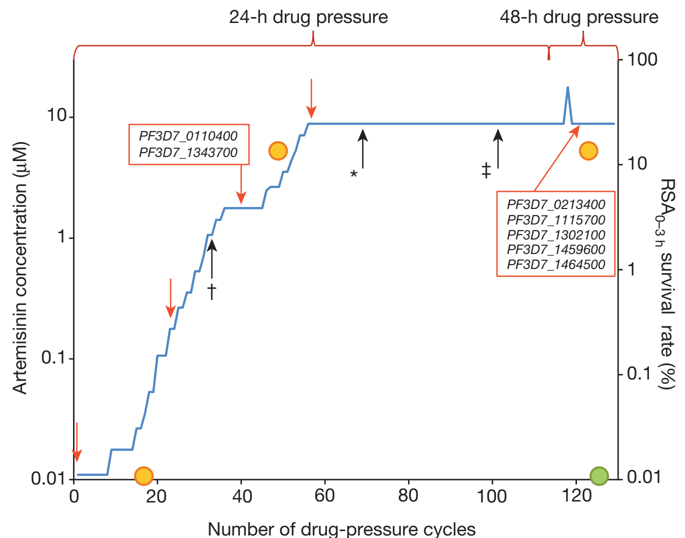

Figure 1 | Temporal acquisition of mutations in F32-ART5. F32-Tanzania parasites exposed to increasing artemisinin concentrations for 120 consecutive cycles[18] were analysed by whole-genome sequencing at five time-points (red arrows). Loci mutated after a given number of drug-pressure cycles are shown (red boxes). The earliest time-points where three mutations were detected by PCR (black arrows) are indicated by † for *PF3D7_1343700*, * for *PF3D7_0213400* and ‡ for *PF3D7_1115700*. Orange and green circles indicate $RSA_{0–3\,h}$ survival rates for F32-ART5 and F32-TEM parasites, respectively (mean of 3 experiments each).

observations and the acquisition of M476I in kelch repeat no. 2 by F32-ART5, we investigated whether K13-propeller polymorphism is a molecular signature of ART resistance in Cambodia.

Emergence and spread of K13-propeller mutants in Cambodia

Over the last decade, the prevalence of ART resistance has steadily increased in the western provinces of Cambodia, but not elsewhere in

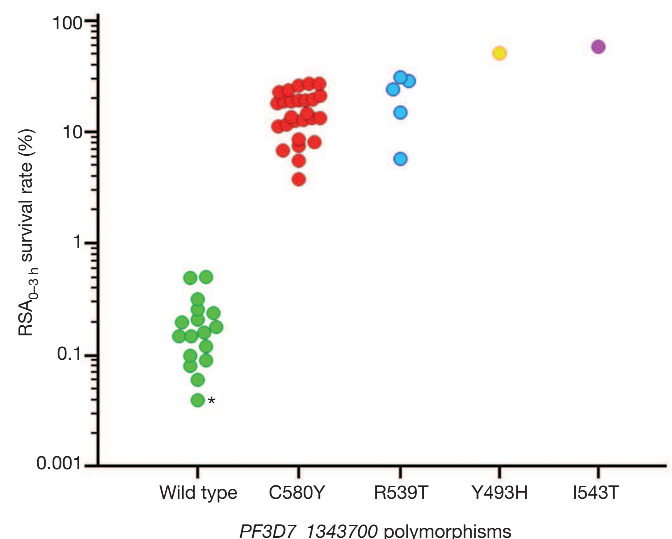

Figure 2 | Survival rates of Cambodian parasite isolates in the $RSA_{0–3\,h}$, stratified by K13-propeller allele. Genotypes were obtained by mining whole-genome sequence data ($n = 21$) or sequencing PCR products ($n = 28$). Mutant parasites have significantly higher $RSA_{0–3\,h}$ survival rates than wild-type parasites: wild type ($n = 17$, median 0.16%, IQR 0.09–0.24, range 0.04–0.51); C580Y ($n = 26$, median 14.1%, IQR 11.3–19.6, range 3.8–27.3, $P < 10^{-6}$ for wild type versus C580Y, Mann–Whitney U test); R539T ($n = 5$, median 24.2%, IQR 12.6–29.5, range 5.8–31.3, $P < 10^{-3}$ for wild type versus R539T); Y493H (51.4%); and I543T (58.0%). The $RSA_{0–3\,h}$ survival rate (0.04%) of control 3D7 parasites is indicated by an asterisk.

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the country[2]. To test whether the spatiotemporal distribution of K13-propeller mutations correlates with that of ART resistance, we sequenced the K13-propeller of archived parasite isolates from Cambodian patients with malaria in 2001–2012 (Extended Data Table 4). Data from six provinces were compared ($n = 886$): Pailin, Battambang and Pursat in the west where ART resistance is established[1,6,8,22], Kratie in the southeast where ART resistance has increased in recent years[2], and Preah Vihear in the north and Ratanakiri in the northeast where there was virtually no evidence of ART resistance during this time period[2]. This analysis reveals overall 17 mutant alleles, including three high-frequency (> 5%) alleles (C580Y, R539T and Y493H). The frequency of wild-type sequence decreased significantly over time in all three western provinces, but not in Preah Vihear or Ratanakiri. The frequency of the C580Y allele increased significantly from 2001–2002 to 2011–2012 in Pailin and Battambang, indicating its rapid invasion of the population and near fixation in these areas (Fig. 3).

To further investigate the geographic diversity of K13-propeller polymorphism in Cambodia, we extended our sequence analysis to include data from four additional provinces ($n = 55$, Kampong Som, Kampot, Mondulkiri and Oddar Meanchey) in 2011–2012 (Extended Data Table 4). Although a large number of mutations are observed (Supplementary Fig. 1 and Extended Data Table 5), the C580Y allele accounts for 85% (189/222) of all mutant alleles observed in 2011–2012 (Extended Data Fig. 2). This mapping outlines the elevated frequency (74%, 222/300) of parasites harbouring a single non-synonymous mutation in the K13-propeller and the geographic disparity of their distribution. Importantly, the frequency distribution of mutant alleles over the various provinces matches that of day 3 positivity in patients treated for malaria with an ACT (Spearman's $r = 0.99$, 95% confidence interval 0.96–0.99, $P < 0.0001$), considered a suggestive sign of clinical ART resistance (Extended Data Fig. 3).

K13-propeller polymorphisms and clinical ART resistance

To confirm that K13-propeller polymorphism is a molecular marker of clinical ART resistance, we first identified 163 patients from Pursat and Ratanakiri in whom we measured parasite clearance half-lives (range 1.58–11.53 h)[6] in 2009–2010 and for which parasites were previously assigned to a KH subpopulation (KH1, KH2, KH3, KH4 or KHA) on the basis of ancestry analysis of whole-genome sequence data[15]. Thirteen patients with mixed genotypes (a wild-type and one or more mutant K13-propeller alleles) were excluded. Of the remaining 150 patients, 72 carried parasites with a wild-type allele and the others carried parasites with only a single non-synonymous SNP in the K13-propeller: C580Y ($n = 51$), R539T ($n = 6$) and Y493H ($n = 21$) (Extended Data Table 6). The parasite clearance half-life in patients with wild-type parasites is significantly shorter (median 3.30 h, interquartile range (IQR) 2.59–3.95) than those with C580Y (7.19 h, 6.47–8.31, $P < 10^{-6}$, Mann–Whitney U test), R539T (6.64 h, 6.00–6.72, $P < 10^{-4}$) or Y493H (6.28 h, 5.37–7.14, $P < 10^{-6}$) parasites (Fig. 4a). Also, the parasite clearance half-life in patients carrying C580Y parasites is significantly longer than those with Y493H parasites ($P = 0.007$, Mann–Whitney U test). These data indicate that C580Y, R539T and Y493H identify slow-clearing parasites in malaria patients treated with ART.

Because KH2, KH3, KH4 and KHA parasites have longer half-lives than KH1 parasites[15], we proposed that allelic variation in the K13-propeller accounts for these differences. Among 150 parasites, 55, 26, 14, 12 and 43 are classified as KH1, KH2, KH3, KH4 and KHA, respectively. Three K13-propeller alleles strongly associate with KH groups: 96% (53/55) of KH1, 96% (25/26) of KH2 and 100% (12/12) of KH4 parasites carry the wild-type, C580Y and Y493H alleles, respectively (Extended Data Table 6). Whereas KH3 parasites ($n = 14$) carry the wild-type, C580Y and R539T alleles, R539T is not observed in KH1, KH2 or KH4

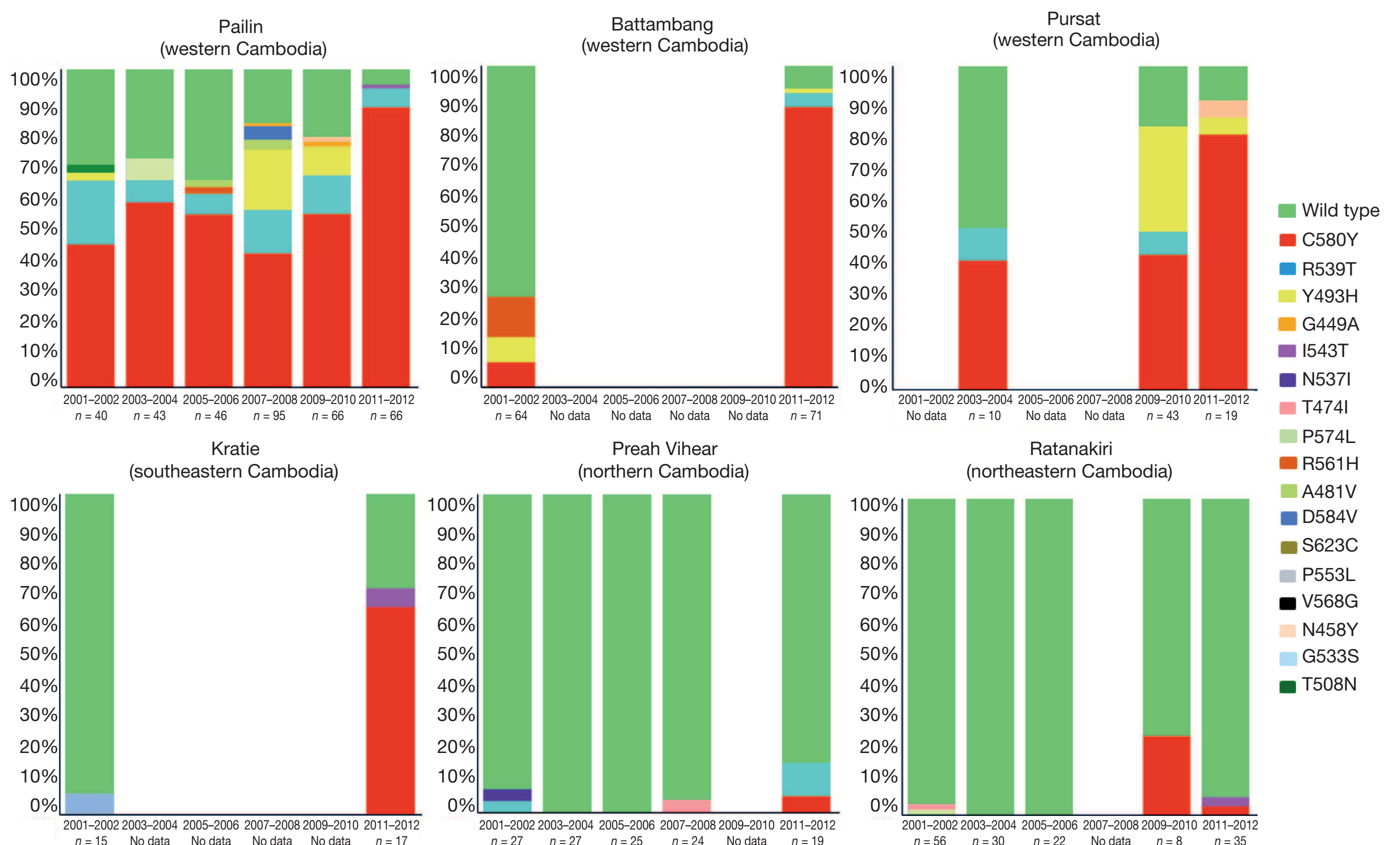

Figure 3 | Frequency of K13-propeller alleles in 886 parasite isolates in six Cambodian provinces in 2001–2012. Genotypes were obtained by sequencing PCR products from archived blood samples. All mutant alleles carry a single non-synonymous SNP (colour-coded, same colour codes as in Fig. 2 for wild type, C580Y, R539T, Y493H and I543T). Significant reductions (Fisher's exact test) in wild-type allele frequencies were observed in Pailin, Battambang, Pursat and Kratie over time (see Methods).

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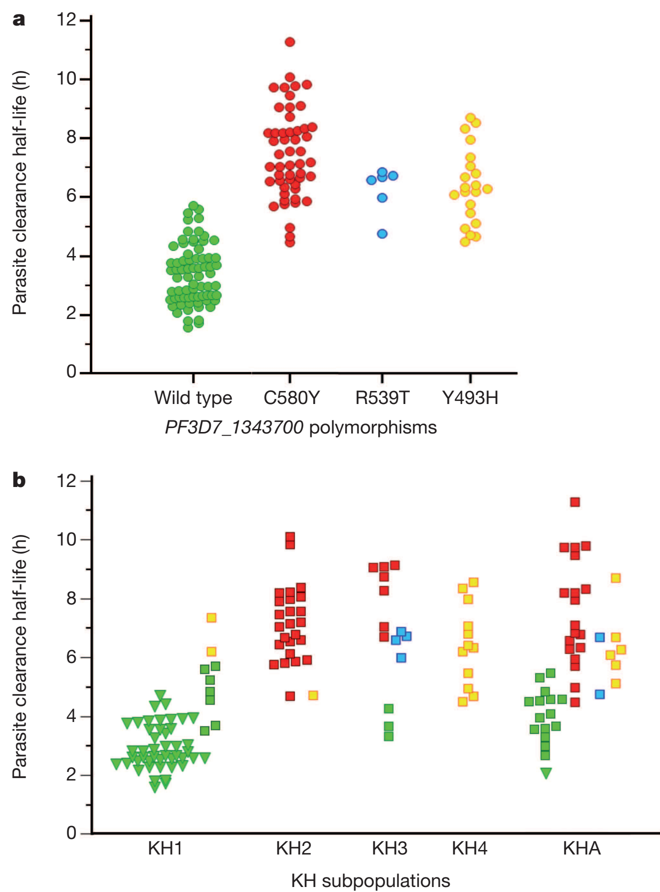

Figure 4 | Parasite clearance half-lives. **a**, Correlation of parasite clearance half-lives and K13-propeller alleles for parasite isolates in Pursat and Ratanakiri in 2009–2010. Wild-type parasites have shorter half-lives (median 3.30 h, IQR 2.59–3.95, $n = 72$) than C580Y (7.19 h, 6.47–8.31, $n = 51$, $P < 10^{-6}$, Mann–Whitney *U* test), R539T (6.64 h, 6.00–6.72, $n = 6$, $P < 10^{-6}$) or Y493H (6.28 h, 5.37–7.14, $n = 21$, $P < 10^{-6}$) parasites. The half-life of C580Y parasites is significantly longer than that of Y493H parasites ($P = 0.007$). **b**, Correlation of parasite clearance half-lives, KH subpopulations[15] and K13-propeller alleles for the same 150 parasite isolates. Half-lives are shown for Pursat (squares) and Ratanakiri (triangles) parasites, stratified by KH group and K13-propeller allele (colour-coded as in **a**). Median half-lives stratified by K13-propeller allele are KH1: wild type (2.88) and Y493H (6.77); KH2: C580Y (7.13) and Y493H (4.71); KH3: wild type (3.65), C580Y (8.73) and R539T (6.65); KH4: Y493H (6.37); and KHA: wild type (4.01), C580Y (7.09), Y493H (6.18) and R539T (5.73).

parasites. As expected, KHA parasites have a mixed allele composition. Importantly, K13-propeller mutations more accurately identify slow-clearing parasites than KH group (Fig. 4b), demonstrating that the association of K13-propeller polymorphism with clinical ART resistance in Cambodia is partially independent of the genetic background of KH subpopulations. Within the KH1 group ($n = 55$), the parasite clearance half-life in patients with wild-type parasites is significantly shorter ($n = 53$, median 2.88 h, IQR 2.52–3.79) than those with Y493H parasites ($n = 2$, median 6.77 h, $P = 0.02$, Mann–Whitney *U* test). Within the KH3 subpopulation ($n = 14$), the half-life in patients with wild-type parasites is shorter ($n = 3$, median 3.65 h) than those with C580Y ($n = 7$, median 8.73 h, IQR 7.35–9.06, $P = 0.02$) or R539T ($n = 4$, 6.65 h, 6.29–6.80, $P = 0.03$) parasites.

Discussion

The F32-ART5 lineage acquired a K13-propeller mutation as it developed ART resistance, as indicated by its ability to survive a pharmacologically relevant exposure to DHA in the $RSA_{0-3\,h}$. Genes putatively associated with ART resistance (*Pfcrt*[23,24], *Pftctp*[25,26], *Pfmdr1*[8,27,28], *Pfmrp1*[27–29] and ABC transporters[30]) or encoding putative targets of ART (*PfATPase6*[31,32] and *Pfubcth*—the orthologue of *Plasmodium chabaudi* ubp1[33,34]) were not mutated during the 5-year selection of F32-ART5, and *Pfmdr1* amplification was not observed[35–40]. In addition, all candidate ART-resistance genes recently identified using population genetics approaches[14,40,41] remained unaltered in F32-ART5, except for *PF3D7_1343700* and *PF3D7_1459600* located in the linkage-disequilibrium windows identified in ref. 16. These findings led us to identify another 17 single K13-propeller mutations in naturally circulating parasites in Cambodia. Several of these mutations associate strongly with the spatiotemporal distribution of ART resistance in Cambodia, increased parasite survival rates in response to DHA *in vitro*, and long parasite clearance half-lives in response to ART treatment *in vivo*. None of the six other genes mutated in F32-ART5 associate with $RSA_{0-3\,h}$ survival rates in parasite isolates from Cambodia.

K13-propeller polymorphism fulfils the definition of a molecular marker of ART resistance for several reasons: (1) there has been a progressive loss of wild-type parasites in western Cambodia during the decade of emerging ART resistance in this region; (2) mutant parasites cluster in Cambodian provinces where ART resistance is well established and are less prevalent where ART resistance is uncommon; (3) *PF3D7_1343700* is located 5.9 kilobases upstream of the 35-kb locus identified in ref. 14 as being under recent positive selection, and within the region of top-ranked signatures of selection outlined in ref. 16; (4) multiple mutations, all non-synonymous, are present in the K13-propeller, reflecting positive selection rather than a hitchhiking effect or genetic drift; (5) mutations occur in a domain that is highly conserved in *P. falciparum*, with only one non-synonymous SNP being documented in a single parasite isolate from Africa[42]; (6) all polymorphisms we observe in Cambodia are novel and all but one (V568G) occur at positions strictly conserved between *Plasmodium* species (Supplementary Fig. 1 and Supplementary Fig. 2), suggesting strong structural and functional constraints on the protein; (7) the three most-prevalent K13-propeller mutations correlate strongly with $RSA_{0-3\,h}$ survival rates *in vitro* and parasite clearance half-lives *in vivo* at the level of individual parasite isolates and malaria patients, respectively; and (8) the frequency of mutant alleles correlates strongly with the prevalence of day 3 positivity after ACT treatment at the level of human populations in Cambodia.

On the basis of homology with other kelch propeller domains, we anticipate that the observed K13-propeller mutations destabilize the domain scaffold and alter its function. The carboxy-terminal portion of *PF3D7_1343700* encodes six kelch motifs, which are found in a large number of proteins with diverse cellular functions[43,44]. Given that the toxicity of ART derivatives depends principally on their pro-oxidant activity, the reported role of some kelch-containing proteins in regulating cytoprotective and protein degradation responses to external stress is particularly intriguing. The K13-propeller shows homology with human KLHL12 and KLHL2, involved in ubiquitin-based protein degradation, and KEAP1, involved in cell adaptation to oxidative stress (Extended Data Fig. 4). More work is needed to delineate the normal function of K13 and the effect of various mutations. Allele exchange studies in mutant and wild-type parasites may help to define the contribution of K13-propeller polymorphisms on different genetic backgrounds to the $RSA_{0-3\,h}$ survival rate. Indeed, it is particularly worrying that as few as two mutations, that is, the K13-propeller M476I and *PF3D7_0110400* D56V, were sufficient to confer ART resistance to F32-Tanzania, which has a typical African genetic background. Cambodian parasites with mutant K13-propellers display a wide range of $RSA_{0-3\,h}$ survival rates (3.8–58%) and parasite clearance half-lives (4.5-11.5 h). Further studies are therefore required to identify additional genetic determinants of ART resistance, which may reside in the strongly selected regions recently identified[14,16]. In this context, analysing the $RSA_{0-3\,h}$ survival rates as a quantitative trait among parasites harbouring the same K13-propeller mutation could help to identify additional genetic loci involved in ART resistance.

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In summary, K13-propeller polymorphism seems to be a useful molecular marker for tracking the emergence and spread of ART-resistant *P. falciparum*.

METHODS SUMMARY

The ART-resistant F32-ART5 parasite line was selected by culturing the ART-sensitive F32-Tanzania clone under a dose-escalating regimen of artemisinin for 5 years. The F32-TEM line was obtained by culturing F32-Tanzania in parallel without artemisinin exposure. Reference DNA was extracted from *P. falciparum* lines 3D7, 89F5 Palo Alto Uganda and K1992. The ring-stage survival assay ($RSA_{0-3\,h}$) was performed as described previously[13]. Whole-genome sequencing was performed on F32-Tanzania, F32-TEM, F32-ART5 (4 time points), three reference strains (3D7, 89F5 and K1992) and 21 Cambodian parasite isolates, using an Illumina paired-reads sequencing technology. A set of 1091 clinical *P. falciparum* isolates was collected from patients participating in ACT efficacy studies in 2001–2012. The K13-propeller was amplified using nested PCR. Double-strand sequencing of PCR products was performed by Macrogen. Sequences were analysed with MEGA 5 software version 5.10 to identify specific SNP combinations. Data were analysed with Microsoft Excel and MedCalc version 12. Differences were considered statistically significant when P values were less than 0.05. Ethical clearances for parasite isolate collections were obtained from the Cambodian National Ethics Committee for Health Research, the Institutional Review Board of the Naval Medical Research Center, the Technical Review Group of the WHO Regional Office for the Western Pacific, and the Institutional Review Board of the National Institute of Allergy and Infectious Diseases.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions B.W., S.M., A.B. and F.B.-V. produced the F32-ART5 and F32-TEM clonal lines and analysed their survival rates. F.A. and J.B. developed computational components of the whole-genome sequence analysis. C.B. and L.M. performed whole-genome sequencing. F.A., C.A., S.K., V.D., P.L., R.L., S.D., Se.S., So.S., C.M.C., D.M.B., W.O.R., B.G., T.F., P.R., J.L.B., R.M.F. and D.M. conducted clinical studies and collected parasite isolates. A.-C.L., N.K., S.K., V.D., S.M. and A.B. performed PCR and sequencing analyses. B.W., F.B.-V., V.D. and D.M. performed *in vitro* assays ($RSA_{0–3\ h}$). O.M. provided genotyping and population structure data for Cambodian parasite isolates. J.-C.B. and O.M.-P. performed three-dimensional structure modelling. F.A., R.M.F., F.B.-V., O.M.-P. and D.M. conceived of the study, supervised the project, processed the data and wrote the manuscript with contributions from B.W., C.A., A.B. and J.-C.B.

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METHODS

Artemisinin- and mock-pressured *P. falciparum* F32 lineages. Mycoplasma-free F32-Tanzania parasites were maintained in human type O red blood cells (RBCs) (Etablisssement Français du Sang) diluted to 2.5% haematocrit in RPMI-1640 medium (Invitrogen, San Diego, CA) supplemented with 5% human serum. Parasite cultures were maintained at 37 °C in an atmosphere of 5% CO_2. Parasitaemia was checked daily and maintained below 10%. For the selection of ART-resistant parasites, asynchronous cultures were adjusted to 5–7% parasitaemia and grown in the presence of escalating doses of artemisinin (from 10 nM to 9 μM) for 24 h for the first 3 years of drug pressure[18]. In the subsequent 2 years, each drug-pressure cycle was done for 48 h with doses ranging from 9 μM to 18 μM. After drug exposure, the medium was discarded and replaced by human-serum-supplemented (20%) drug-free medium. Parasitaemia was monitored daily until it reached 5%. At that time, drug pressure was reapplied. The parasite line obtained after an effective 5 years of discontinuous ART pressure was named F32-ART5. In parallel, the parental F32-Tanzania line was kept as a control in continuous culture for the same time under the same conditions (that is, RBCs, serum and media) but without artemisinin exposure. The resulting control line was called F32-TEM.

Laboratory-adapted *P. falciparum* lines. Reference DNA was extracted from the laboratory-adapted *P. falciparum* lines 3D7 (MR4, Manassas, VA), 89F5 Palo Alto Uganda (a clone from the Palo Alto line, originating from Uganda in 1978, which displayed high susceptibility to artemether treatment in the *Saimiri sciureus* experimental host (O. Mercereau-Puijalon, H. Contamin and J.-C. Barale, unpublished data)) and K1992, an isolate collected in Pailin in 1992 before the mass deployment of ART in that area (provided by the French National Reference Center of Malaria). Parasite DNA was extracted from frozen blood aliquots (200 μl) using the Mini blood kit (Qiagen) according to the manufacturer's instructions.

Culture-adapted *P. falciparum* isolates from Cambodia. Fifty clinical *P. falciparum* isolates from Cambodia (collected in 2010 and 2011) were adapted to *in vitro* culture as described in ref. 45. Their geographic origin is indicated in Supplementary Table 1. Parasite clearance rates were not determined for these patient isolates, as they were collected during field trials that did not include such measurements. Parasite DNA was extracted from frozen blood aliquots (200 μl) using the Mini blood kit (Qiagen).

Ring-stage survival assay. The ring-stage survival assay ($RSA_{0-3\,h}$) was carried out as described in ref. 13 using highly synchronous parasite cultures. In brief, 0–3 h post-invasion ring-stage parasites were exposed to 700 nM DHA (dihydroartemisinin, obtained from WWARN (http://www.wwarn.org/research/tools/qaqc)) or its solvent DMSO for 6 h, washed and then cultivated for the next 66 h without drug. Survival rates were assessed microscopically by counting in Giemsa-stained thin smears the proportion of viable parasites that developed into second-generation rings or trophozoites with normal morphology.

Ethical clearance. Ethical clearances for the collection of parasite isolates from patients were obtained from the Cambodian National Ethics Committee for Health Research, the Institutional Review Board of the Naval Medical Research Center, the Technical Review Group of the WHO Regional Office for the Western Pacific, and the Institutional Review Board of the National Institute of Allergy and Infectious Diseases. Work was conducted in compliance with all relevant ethical standards and regulations governing research involving human subjects. Written informed consent was obtained from all adult participants or the parents or guardians of children.

Temporal and geographical sample collection. A set of 941 clinical *P. falciparum* isolates was collected from patients participating in therapeutic efficacy studies of ACTs, conducted as part of the routine antimalarial drug efficacy monitoring of Cambodia's National Malaria Control Program from 2001 to 2012, and from studies conducted by NAMRU-2 (Extended Data Table 4). Venous blood samples (5 ml) collected in EDTA or ACD were transported to Institut Pasteur du Cambodge in Phnom Penh within 48 h of collection at 4 °C and then kept at −20 °C until DNA extraction. Parasite DNA was extracted from frozen blood aliquots (200 μl) using the Mini blood kit (Qiagen).

Measurement of parasite clearance half-life. Patients with uncomplicated or severe *P. falciparum* malaria and initial parasite density $\geq 10{,}000\ \mu l^{-1}$ were enrolled in Pursat and Ratanakiri provinces in 2009 and 2010 as described[6,13]. Patients were treated with an ART and their parasite density measured every 6 h from thick blood films until parasitaemia was undetectable. The parasite clearance half-life in 163 patients was derived from these parasite counts using WWARN's on-line Parasite Clearance Estimator (http://www.wwarn.org/toolkit/data-management/parasite-clearance-estimator). The study is registered at ClinicalTrials.gov (number NCT00341003).

Whole-genome sequencing of parasite DNA. Whole-genome sequencing was performed on F32-Tanzania, F32-TEM, the F32-ART5 lineage (4 time-points), three reference strains (3D7, 89F5 and K1992) and 21 parasite isolates from Cambodia, using an Illumina paired-reads sequencing technology. Illumina library preparation and sequencing followed standard protocols developed by the supplier. Briefly, genomic DNA was sheared by nebulization, and sheared fragments were end-repaired and phosphorylated. Blunt-end fragments were A-tailed, and sequencing adapters were ligated to the fragments. Inserts were sized using Agencourt AMPure XP Beads (± 500 bp; Beckman Coulter Genomics) and enriched using 10 cycles of PCR before library quantification and validation. Hybridization of the library to the flow cell and bridge amplification was performed to generate clusters, and paired-end reads of 100 cycles were collected on a HiSeq 2000 instrument (Illumina). After sequencing was complete, image analysis, base calling and error estimation were performed using Illumina Analysis Pipeline version 1.7.

Raw sequence files were filtered using Fqquality tool, a read-quality filtering software developed by N. Joly, which enables the trimming of the first and last low-quality bases in reads. The trimmed reads from controlled Fastq files were mapped on a reference genome (*P. falciparum* 3D7) with the Burrows-Wheeler Alignment (BWA), generating a BAM file (a binary file of tab-delimited format SAM). Next, we used Samtools to prepare a pileup file, which was formatted using in-house software to implement the data into the Wholegenome Data Manager (WDM) database (Beghain *et al.*, in preparation). WDM software is designed to compare and/or align partial or whole *P. falciparum* genomes.

Sequencing genes containing non-synonymous SNPs in F32-ART5. PCR amplification of selected genes was performed using the primers listed in Extended Data Table 1. Two μl of DNA was amplified with 1 μM of each primer, 0.2 mM dNTP (Solis Biodyne), 3 mM $MgCl_2$ and 2 U *Taq* DNA polymerase (Solis Biodyne), using the following cycling program: 5 min at 94 °C, then 40 cycles of 30 s at 94 °C, 90 s at 60 °C, 90 s at 72 °C and final extension 10 min at 72 °C. PCR products were detected by 2% agarose gel electrophoresis and ethidium bromide staining. Double-strand sequencing of PCR products was performed by Beckman Coulter Genomics. Sequences were analysed with MEGA 5 software version 5.10 in order to identify specific SNP combinations.

Sequencing the K13-propeller domain. The K13-propeller domain was amplified using the following primers: for the primary PCR (K13-1 5′-cggagtgaccaaatctggga-3′ and K13-4 5′-gggaatctggtggtaacagc-3′) and the nested PCR (K13-2 5′-gccaagctg ccattcatttg -3′ and K13-3 5′-gccttgttgaaagaagcaga -3′). One μl of DNA was amplified with 1 μM of each primer, 0.2 mM dNTP (Solis Biodyne), 3 mM $MgCl_2$ and 2 U *Taq* DNA polymerase (Solis Biodyne), using the following cycling program: 5 min at 94 °C, then 40 cycles of 30 s at 94 °C, 90 s at 60 °C, 90 s at 72 °C and final extension 10 min at 72 °C. For the nested PCR, 2 μl of primary PCR products were amplified under the same conditions, except for the $MgCl_2$ concentration (2.5 mM). PCR products were detected using 2% agarose gel electrophoresis and ethidium bromide staining. Double-strand sequencing of PCR products was performed by Macrogen. Sequences were analysed with MEGA 5 software version 5.10 to identify specific SNP combinations.

Deep-sequencing of clinical parasite isolates and population structure analysis. DNA extraction, Illumina sequencing and SNP genotyping of clinical parasite isolates obtained from malaria patients in Pursat and Ratanakiri provinces, Cambodia, have been previously described[15]. Population structure analysis of these parasites identified four subpopulations: KH1, KH2, KH3 and KH4. Parasites with <80% ancestry from any of these four groups were deemed admixed (KHA).

Temporal acquisition of mutations in the F32-ART5 lineage. The F32-ART5 lineage was explored by whole-genome sequencing using samples collected at time 0 (original F32-Tanzania clonal line), day 196 (0.2-μM pressure cycle no. 23), day 385 (1.8-μM pressure cycle no. 39), day 618 (9-μM pressure cycle no. 56) and day 2,250 (9-μM pressure cycle no. 120). The F32-TEM sample was collected on day 2,250. Additional samples collected at the time of the 30th, 33rd, 34th, 36th, 68th and 98th pressure cycles were studied by PCR. DNA from parasite cultures was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturer's instructions.

The F32-ART5 samples tested in the ring-stage survival assay ($RSA_{0-3\,h}$) were collected at the time of the 17th, 48th and 122nd pressure cycles (0.04, 2.7 and 9 μM ART), respectively. The F32-TEM sample was collected at the last mock pressure cycle. The $RSA_{0-3\,h}$ survival rates were determined in triplicate experiments with different batches of red blood cells, and evaluated as above using Giemsa-stained thin smears read by two independent microscopists (B.W. and F.B.-V.). Survival rates were compared using Mann–Whitney *U* test. The $RSA_{0-3\,h}$ survival rates of the F32-ART5 samples were as follows: at drug-pressure cycles: no. 17 ($n = 3$, median 0%, IQR 0–0.07), no. 48 ($n = 3$, median 11.7%, IQR 10.3-14.6; $P = 0.04$ for no. 17 versus no. 48, Mann–Whitney *U* test) and no. 122 ($n = 3$, median 12.8%, IQR 10.6-14.5, $P = 0.04$ and $P = 0.82$ for no. 17 versus no. 122 and no. 48 versus no. 122). The $RSA_{0-3\,h}$ survival rate of the F32-TEM line was also determined in triplicate experiments ($n = 3$, median 0%, IQR 0-0.05, $P = 0.81$ for TEM versus no. 17, Mann–Whitney *U* test).

Prevalence of K13-propeller mutations in 886 clinical parasite isolates collected in six Cambodian provinces in 2001–2012. The K13-propeller was genotyped by

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sequencing PCR products amplified from 886 archived blood samples. The number of samples analysed from each province each year is indicated in Fig. 3. Fisher's exact test was used to compare the frequency of isolates harbouring a wild-type K13-propeller sequence in each province over time. A significant decrease of the frequency of the wild-type K13-propeller allele was observed in the western provinces during the decade. In Pailin, it decreased from 30.0% in 2001–2002 (12/40) to 4.8% in 2011–2012 (4/84), $P = 0.0002$, in Battambang from 71.9% in 2001–2002 (46/64) to 7.0% in 2011–2012 (5/71), $P < 10^{-6}$, in Pursat from 50.0% in 2003–2004 (5/10) to 10.5% in 2011–2012 (2/19), $P = 0.03$; and in Kratie from 93.3% in 2001–2002 (14/15) to 29.4% in 2011–2012 (5/17), $P = 0.0003$. Significant decreases in wild-type allele frequency were not observed in Preah Vihear (from 92.6% in 2001–2002 (25/27) to 84.2% in 2011–2012 (16/19), $P = 0.63$); or Ratanakiri (from 96.4% in 2001–2002 (54/56) to 94.3% in 2011–2012 (33/35), $P = 0.63$). The frequency of C580Y increased in Pailin from 45.0% (18/40) in 2001–2002 to 88.1% (74/84) in 2011–2012 ($P < 10^{-6}$), and in Battambang from 7.8% (5/64) in 2001–2002 to 87.3% (62/71) in 2011–2012 ($P < 10^{-6}$) indicating its rapid invasion of the population and near fixation in these provinces.

Three-dimensional structure modelling of the K13-propeller. The 3D-structural model of the kelch propeller domain of *PF3D7_1343700* ('K13-propeller') was obtained by homology modelling satisfying spatial restraints using Modeller v9.11 (http://modbase.compbio.ucsf.edu). The 295 amino acids composing the K13-propeller are 22%, 25% and 25% identical to the kelch propeller domain of the human KEAP1 (Protein Data Bank (PDB; http://www.rcsb.org/) 2FLU), KLHL12 (PDB 2VPJ) and KLHL2 (PDB 2XN4) proteins, respectively, that were used as templates to model the 3D-structure of the K13-propeller. The reliability of the obtained model was assessed using two classical criteria. First, the significance of the sequence alignment between the K13-propeller domain and one template was confirmed by an E-value $= 0$, as calculated by Modeller using the Built-Profile routine. Second, the model achieved a GA341 model score $= 1$ (a score ≥ 0.7 corresponds to highly reliable models). Localization of the mutants in the K13-propeller 3D-model was prepared using the PyMOL Molecular Graphics System, version 1.5.0.4 (Schrödinger; http://www.pymol.org).

Statistical analysis. Data were analysed with Microsoft Excel and MedCalc version 12. Quantitative data were expressed as median, interquartile range (IQR). The Mann–Whitney U test (independent samples, two-sided) was used to compare two groups, and the Kruskal–Wallis test (H-test, two-sided) was used to compare more than two groups. The Spearman's rho rank correlation coefficient (and the 95% confidence interval for the correlation coefficient) was used to measure the strength of relationship between the prevalence of wild-type K13-propeller allele and the frequency of day 3 positivity (defined as persistence of microscopically detectable parasites on the third day of artemisinin-based combination therapy)[2]. Fisher's exact test was used to compare frequency data and the Clopper–Pearson exact method based on the beta distribution was used to determine 95% confidence intervals for proportions. Differences were considered statistically significant when P values were less than 0.05.

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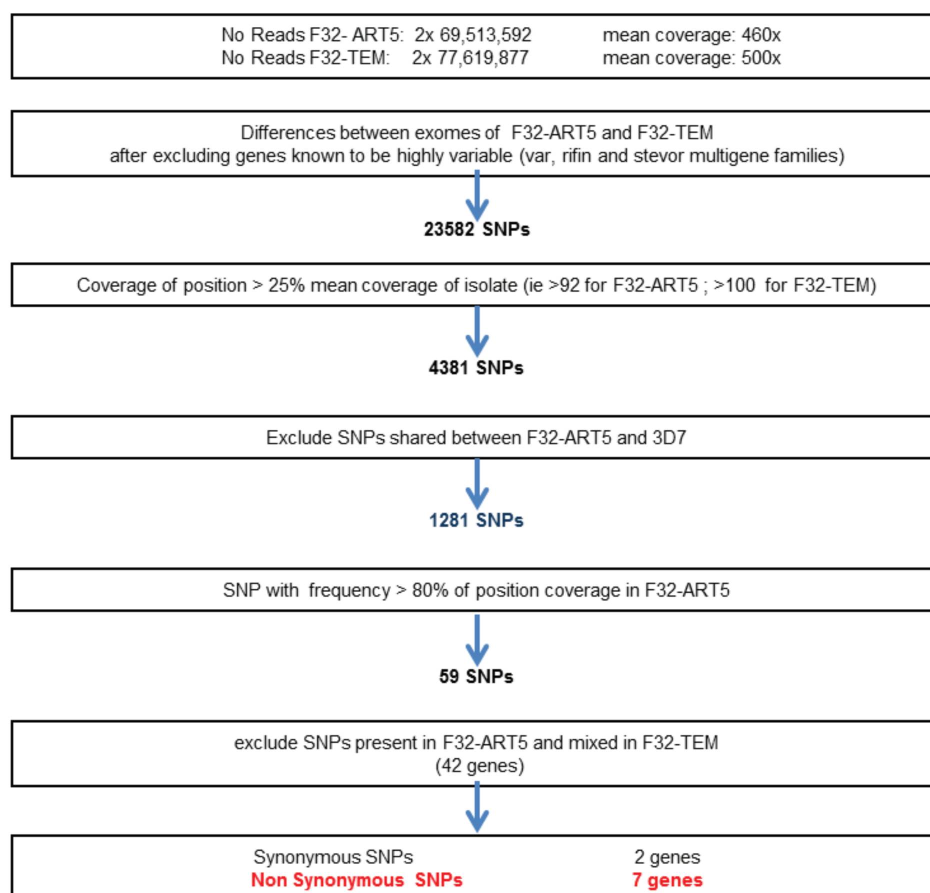
a



b

Chromosome	Position	Gene ID	F32-ART5 coverage	F32-ART5 No reads with mutant SNP	F32-ART5 % reads with mutant SNP	F32-TEM coverage	F32-TEM No reads with w.t. SNP	F32-TEM % reads with w.t. SNP
1	394452	PF3D7_0110400;DNA-directed RNA polymerase 2, putative	224	222	99.11	335	334	99.70
2	542625	PF3D7_0213400;protein kinase 7 (PK7)	242	242	100.00	403	403	100.00
11	593379	PF3D7_1115700;cysteine proteinase falcipain 2a	234	231	98.72	290	289	99.66
13	121689	PF3D7_1302100;gamete antigen 27/25 (Pfg27)	261	259	99.23	343	342	99.71
13	1725570	PF3D7_1343700;kelch protein, putative	1004	1004	100.00	1161	1160	99.91
14	2442240	PF3D7_1459600;conserved Plasmodium protein, unknown	165	142	86.06	225	225	100.00
14	2612177	PF3D7_1464500;conserved Plasmodium membrane protein, unknown	401	399	99.50	428	428	100.00

Extended Data Figure 1 | SNP-calling algorithm and sequence and coverage of SNPs. a, SNP-calling algorithm of the whole-genome sequence comparison of F32-ART5 and F32-TEM. **b**, Sequence and coverage of SNPs in seven candidate genes differing in F32-TEM and F32 ART5.

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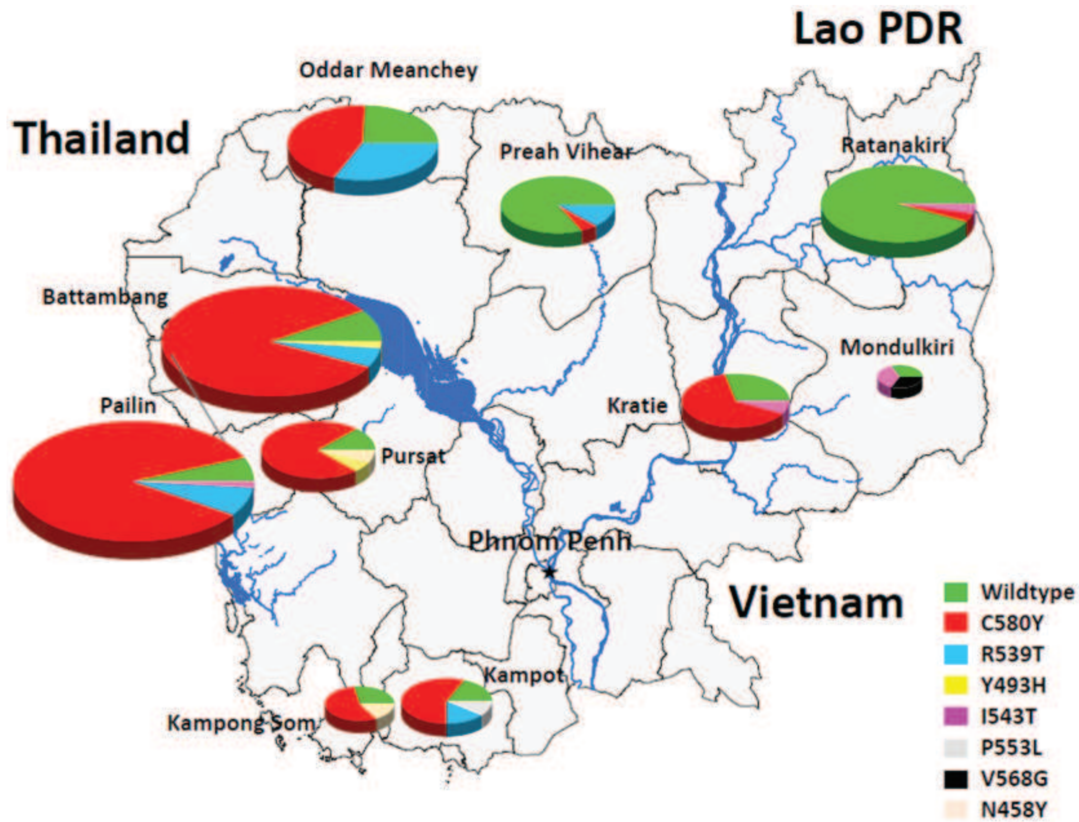

Extended Data Figure 2 | Geographic distribution of K13-propeller alleles in Cambodia in 2011–2012. Pie charts show K13-propeller allele frequencies among 300 parasite isolates in ten Cambodian provinces. Pie sizes are proportional to the number of isolates and the different alleles are colour-coded as indicated. The frequencies (95% confidence interval) of mutant K13-propeller alleles are: Pailin (95%, 88–99, $n = 84$), Battambang (93%, 87–99, $n = 71$), Pursat (89%, 67–99, $n = 19$), Kampot (83%, 52–98, $n = 12$), Kampong Som (71%, 29–96, $n = 7$), Oddar Meanchey (76%, 58–89, $n = 33$), Preah Vihear (16%, 3–40, $n = 19$), Kratie (71%, 44–90, $n = 17$), Mondulkiri (67%, 9–99, $n = 3$) and Ratanakiri (6%, 1–19, $n = 35$).

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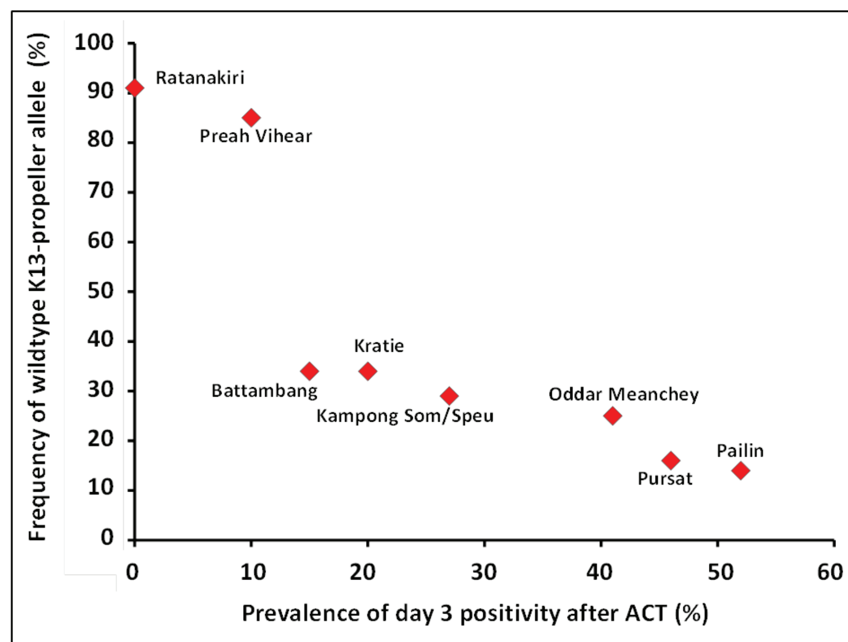

Extended Data Figure 3 | Correlation between the frequency of wild-type K13-propeller alleles and the prevalence of day 3 positivity after ACT treatment in eight Cambodian provinces. The frequency of day 3 positivity is plotted against the frequency of wild-type K13-propeller alleles. Data are derived from patients treated with an ACT for *P. falciparum* malaria in 2010–2012 in eight Cambodian provinces (Extended Data Figure 2): Pailin ($n = 86$, 2011 WHO therapeutic efficacy study, artesunate-mefloquine); Pursat ($n = 32$, 2012 WHO therapeutic efficacy study, dihydroartemisinin-piperaquine); Oddar Meanchey ($n = 32$, 2010 NAMRU-2 therapeutic efficacy study, artesunate-mefloquine); Kampong Som/Speu ($n = 7$, 2012 WHO therapeutic efficacy study, dihydroartemisinin-piperaquine); Battambang ($n = 18$, 2012 WHO therapeutic efficacy study, dihydroartemisinin-piperaquine); Kratie ($n = 15$, 2011 WHO therapeutic efficacy study, dihydroartemisinin-piperaquine); Preah Vihear ($n = 19$, 2011 WHO therapeutic efficacy study, dihydroartemisinin-piperaquine); Ratanakiri ($n = 32$, 2010 WHO therapeutic efficacy study, dihydroartemisinin-piperaquine). Spearman's coefficient of rank correlation (8 sites): $r = -0.99$, 95% confidence interval -0.99 to -0.96, $P < 0.0001$.

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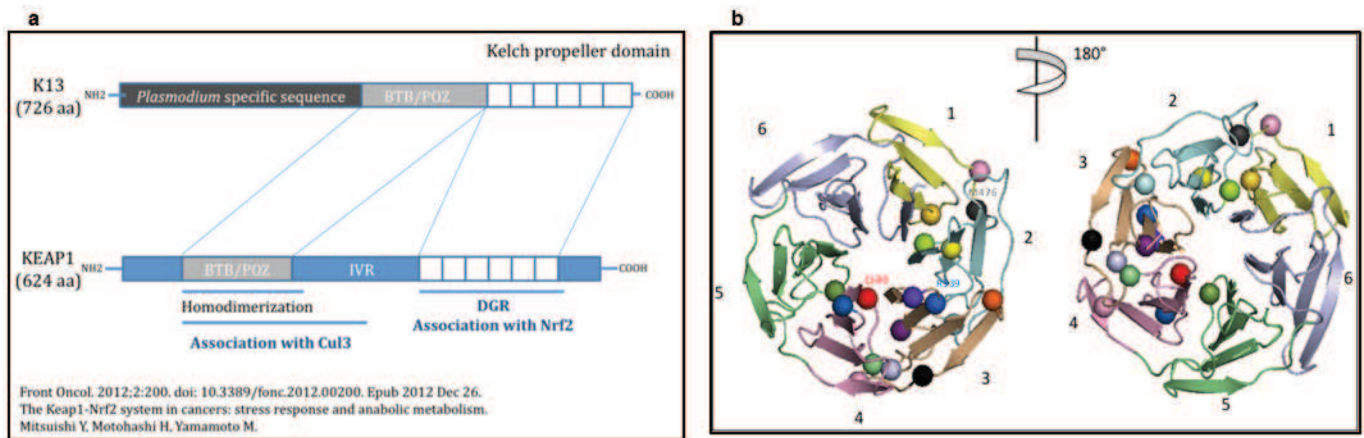

Extended Data Figure 4 | Schematic representation of homology between *P. falciparum* K13 and human KEAP1 proteins and structural 3D model of the K13-propeller domain. **a**, Schematic representation of the predicted *PF3D7_1343700* protein and homology to human KEAP1. Similar to KEAP1, *PF3D7_1343700* contains a BTB/POZ domain and a C-terminal 6-blade propeller, which assembles kelch motifs consisting of four anti-parallel beta sheets. **b**, Structural 3D model of the K13-propeller domain showing the six kelch blades numbered 1 to 6 from N to C terminus and colour-coded as in Supplementary Fig. 1. The level of amino-acid identity between the K13-propeller and kelch domains of proteins with solved 3D structures, including human KEAP1[46,47], enabled us to model the 3D structure of the K13-propeller and to map the mutations selected under ART pressure (Extended data Table 5). The accuracy of the K13-propeller 3D model was confirmed by Modeller-specific model/fold criteria of reliability (see Methods). We predict that the K13-propeller folds into a 6-bladed β-propeller structure[48] closed by the interaction between a C-terminal beta-sheet and the N-terminal blade[46,48]. The first domain has three β-sheets, the fourth one being contributed by an extra C-terminal β-sheet called β'1 in Supplementary Fig. 1. The human KEAP1 kelch propeller scaffold is destabilized by a variety of mutations affecting intra- or inter-blade interactions in human lung cancer[46] and hypertension[47]. The positions of the various mutations are indicated by a sphere, colour-coded as in Figs 2–4. The M476 residue mutated in F32-ART5 is indicated in dark grey. Like the mutations observed in human KEAP1[46,47], many K13-propeller mutations are predicted to alter the structure of the propeller or modify surface charges, and as a consequence alter the biological function of the protein. Importantly, the two major mutations C580Y (red) and R539T (blue) observed in Cambodia are both non-conservative and located in organized secondary structures: a β-sheet of blade 4 where it is predicted to alter the integrity of this scaffold and at the surface of blade 3, respectively. The kelch propeller domain of KEAP1 is involved in protein–protein interactions like most kelch containing modules[43]. KEAP1 is a negative regulator of the inducible Nrf2-dependent cytoprotective response, sequestering Nrf2 in the cytoplasm under steady state. Upon oxidative stress, the Nrf2/KEAP1 complex is disrupted, and Nrf2 translocates to the nucleus, where it induces transcription of cytoprotective ARE-dependent genes[49,50]. We speculate that similar functions may be performed by *PF3D7_1343700* in *P. falciparum*, such that mutations of the K13-propeller impair its interactions with an unknown protein partner, resulting in a deregulated anti-oxidant/cytoprotective response. The *P. falciparum* anti-oxidant response is maximal during the late trophozoite stage, when haemoglobin digestion and metabolism are highest[51]. Its regulation is still poorly understood and no Nrf2 orthologue could be identified in the *Plasmodium* genome.

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Extended Data Table 1 | Sequence of the primers used to amplify the genes containing nonsynonymous single-nucleotide polymorphisms in F32-ART5

Targeted gene	Primer forward sequence	Primer reverse sequence
PF3D7_0110400	5'-ttgagcttctttttcccaataatggc-3'	5'-tgatatatgtttgtaggagctgtgag-3'
PF3D7_0213400	5'-gtgaaaaggataataaattctatgcc-3'	5'-tatctaccatatattctgattctcc-3'
PF3D7_1115700	5'-agcaagaacgttttgtgtaaa-3'	5'-gaattctttaatggttttgaagat-3'
PF3D7_1302100	5'-taatatgtaaagtgattatgtatatcgc-3'	5'-atgctagagaagttaaagagaagaagcg-3'
PF3D7_1343700	5'-agaagagccatcatatccccc-3'	5'-agtggaagacatcatgtaaccag-3'
PF3D7_1459600	5'-atatgagtaaaatgtcaggttttgg-3'	5'-tgcttgttgtgattcatgggg-3'
PF3D7_1464500	5'-aaatagttgggcgtagctcag-3'	5'-tatcacaattaagtgtatcacaacg-3'

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Extended Data Table 2 | Description of the eight nonsynonymous, single-nucleotide polymorphisms acquired in the F32-ART5 compared to the F32-TEM lineage during an effective 5-year discontinuous exposure to increasing concentrations of artemisinin

Gene ID (Plasmodb 9.1)	Annotation	Chromosome/ position mutated	Nucleotide position in coding sequence	F32-TEM codon[#]	Codon F32-ART5 lineage					
					Day 0	Drug pressure cycle #				Mutant codon
						23	39	56	120	
					-	0.2 µM ART*	1.8 µM ART*	9 µM ART*	9 µM ART*	
PF3D7_0110400	DNA-directed RNA polymerase 2 complex subunit RPB9, putative	01/39452	173	gAt	gAt	gAt	**gTg**	**gTg**	**gTg**	**D56V**
PF3D7_1343700[a]	kelch protein, putative, called here 'K13'	13/1725570	1428	atG	atG	atG	**atA**	**atA**	**atA**	**M476I**
PF3D7_0213400	protein kinase 7 (PK7)	02/542625 02/542627	310 312	GaA	GaA	GaA	GaA	GaA	**TaG**	**E104stop**
PF3D7_1115700	cysteine proteinase falcipain 2a	11/593378	206	tCa	tCa	tCa	tCa	tCa	**tGa**	**S69stop**
PF3D7_1302100	gamete antigen 27/25 (Pfg27)	13/121689	601	Cca	Cca	Cca	Cca	Cca	**Aca**	**P201T**
PF3D7_1459600[a]	conserved *Plasmodium* protein, unknown function	14/2442240	896	aGt	aGt	aGt	aGt	aGt	**aCt**	**S299T**
PF3D7_1464500	conserved *Plasmodium* membrane protein, unknown function	14/2612177	4886	aAt	aAt	aAt	aAt	aAt	**aGt**	**N1629S**

3D7-type sequence; the same codon sequence is also observed in the parental F32-Tanzania line.
* Artemisinin (ART) dose used for selection during the corresponding drug-pressure cycle.
[a]Genes found in the chromosomal location of top-ranked signatures of selection in ref. 16.

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Extended Data Table 3 | Reported characteristics of the genes mutated in F32-ART5 parasites

PF3D7_0110400 (PFA0505c), is a two-exon gene, codes for the RNA Polymerase II subunit 9 (RPB9), a small integral Pol II subunit, which is highly conserved among eukaryotes. The yeast RPB9 ortholog has been shown to have a role in assuring the fidelity of transcription *in vivo*. Deletion of the gene results in error-prone transcription[52]. The protein has a predicted zinc ribbon domain similar to the zinc ribbon domain of TFIIS (RNA Polymerase II elongation factor) that contains the essential catalytic Asp-Glu dipeptide[53]. Very little is known on the protein in *Plasmodia*, although the gene is expressed and the protein is present in blood stages (www. plasmodb.org). It is difficult to make any prediction on the possible phenotypic consequences of the D56V mutation, which is located in a *Plasmodium*-specific, well-conserved domain.

PF3D7_1343700 (PF13_0238), is a one-exon gene (called here K13) that codes for a putative kelch protein. K13 has a predicted 3-domain structure, with an approx. 225 residue long, *Plasmodium*-specific and well conserved N-terminal domain, followed by a BTB/ POZ domain and a 6-blade C-terminal propeller domain formed of canonical kelch motifs[43,48]. Little is known about the protein in malaria parasites. Proteomics data indicate that it is produced by asexual (trophozoites, schizonts, merozoites and rings) and sexual blood stages (gametocytes) of *P. falciparum*, and that it possesses phosphorylated residues in the N-terminal *Plasmodium*-specific domain (www. plasmodb.org). The M476I mutation is located between the first and second blade of the propeller domain.

PF3D7_0213400 (PFB0605w), is a four-exon gene that codes for protein kinase 7 (PK7) expressed during the asexual blood stage development, in gametocytes and ookinetes. The E104 stop mutation (two SNPs affecting the same codon) observed in F32-ART5 interrupts the gene resulting in a truncated putative translation product lacking more than 2/3 of its sequence. Studies with genetically inactivated parasites have shown that PK7-KO *P. falciparum* parasites have an asexual growth defect due to a reduced number of merozoites per schizont[54]. Furthermore, PK7 is important for mosquito transmission, with a collapsed number of ookinetes in *P. falciparum*[54] and in *P. berghei*, where no sporoblasts and consequently no sporozoites are formed[55]. This transmission defective phenotype is unlikely to survive in the field.

PF3D7_1115700 (PF11_0165), is a one-exon gene that codes for falcipain 2a, a cysteine proteinase produced by maturing blood stages (trophozoites and schizonts) and involved in hemoglobin degradation[56]. The S69stop mutation located in the pro-enzyme region precludes expression of an active enzyme by F32-ART5 parasites. Gene inactivation has shown to induce a transient reduction of hemoglobin degradation compensated by expression of other members of the cysteine proteinases family, with minimal impact on growth rate[57,58]. However, *falcipain 2a* is the only gene from the list of seven affected loci that has been associated with the in vitro response to artemisinin. Indeed, it has been convincingly shown that inhibition of falcipain2a-dependent hemoglobin digestion by specific inhibitors or by gene inactivation reduced parasite susceptibility to artemisinins[19]. Moreover, ring stages that do not massively digest hemoglobin display a reduced susceptibility to artemisinins[59].

PF3D7_1302100 (PF13_0011), is a one-exon gene that codes for the gamete antigen 27/25 (Pfg27) produced at the onset of gametocytogenesis. The gene is specific to *P. falciparum* and its close relatives such as *P. reichnowi*. This is an abundant, dimeric phosphorylated cytoplasmic protein that binds RNA. The various KO lines generated display conflicting phenotypes some being deficient in gametocyogenesis[60], while other Pfg27-defective lines undergo unimpaired gametocytogenesis up to stage V, mature gametocytes although absence of Pfg27 is associated with abnormalities in intracellular architecture of gametocytes[61]. The crystal structure shows that the protein forms a dimer, displays a particular RNA binding fold and possesses two Pro-X-X-Pro motifs (known ligands for various domains, including SH3 modules), which combine to form a receptacle for SH3 modules[62]. The P201T mutation is located in the C-terminal ProX-X-Pro motif and predicted to alter the spatial structure of the interaction domain and thus have functional consequences.

PF3D7_1459600 (PF14_0569), is a two-exon gene that codes for a 806 residue-long, conserved protein of unknown function. The *P. yoelii* ortholog has been annotated as the CAAT-box DNA binding subunit B. Close orthologs can be found only among the *Plasmodium* species. Proteomics data indicate that the protein is present in asexual (trophozoites, schizonts, merozoites and rings) and sexual (gametocytes) blood stages of *P. falciparum*. A predicted approx. 130 aa-long Interpro domain suggests presence of an N-terminal multi-helical, alpha-alpha 2-layered structural VHS fold, possibly involved in intracellular membrane trafficking. The rest of the coding sequence carries no specific domain signature. The S299T mutation is located within this "unknown" region.

PF3D7_1464500 (PF14_0603), is a five-exon gene that codes for a 3251 residue-long protein of unknown function, with 4 predicted trans-membrane domains, but otherwise no specific domain signature. Apart from proteomics data indicating its expression and phosphorylation in schizonts**,** with possible expression in gametocytes and sporozoites as well, little is known about its putative function. The N1629S mutation is located in the middle of the protein, with unpredictable phenotypic impact.

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Extended Data Table 4 | Geographic origin and year of collection of archived blood samples studied for K13-propeller polymorphism

Region	Province	Year of collection						Total
		2001-2002	2003-2004	2005-2006	2007-2008	2009-2010	2011-2012	
Western Cambodia	Battambang	64	0	0	0	0	71	**135**
	Pailin	40	43	46	95	66	84	**374**
	Pursat	0	10	0	0	43	19	**72**
Southern Cambodia	Kampot	0	0	0	0	0	12	**12**
	Kampong Som	0	0	0	0	0	7	**7**
Northern Cambodia	Oddar Meanchey	0	0	0	0	0	33	**33**
	Preah Vihear	27	27	25	24	0	19	**122**
Eastern Cambodia	Kratie	15	0	0	0	0	17	**32**
	Mondulkiri	0	0	0	0	0	3	**3**
	Ratanakiri	56	30	22	0	8	35	**151**
Total		**202**	**110**	**93**	**119**	**117**	**300**	**941**

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Extended Data Table 5 | Polymorphisms observed in the K13-propeller in Cambodian *P. falciparum* isolates collected in 2001–2012 and in The Gambia (ref. 42)

Codon Position	Amino Acid reference	Nucleotide reference	Amino Acid mutation	Nucleotide mutation
449	G	g**g**t	A	g**C**t
458	N	**a**at	Y	**T**at
474	T	a**c**a	I	a**T**a
476*	M	at**g**	I	at**A**
481	A	g**c**t	V	g**T**t
493	Y	**t**ac	H	**C**ac
508	T	**a**ct	N	a**A**t
527	P	**c**ct	T	**A**ct
533	G	**g**gt	S	**A**gt
537	N	a**a**t	I	a**T**t
539	R	a**g**a	T	a**C**a
543	I	a**t**t	T	a**C**t
553	P	c**c**g	L	c**T**g
561	R	c**g**t	H	c**A**t
568	V	g**t**g	G	g**G**g
574	P	c**c**t	L	c**T**t
580	C	t**g**t	Y	t**A**t
584	D	g**a**t	V	g**T**t
612**	E	ga**a**	D	ga**T**
623	S	**a**gt	C	**T**gt

* Observed in F32-ART5, not observed in Cambodia
** Reported in The Gambia[42], not observed in Cambodia

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Extended Data Table 6 | Association between polymorphisms observed in the K13-propeller and KH subpopulations (ref. 15) in 150 *P. falciparum* isolates collected in 2009–2010 in Pursat (*n* = 103) and Ratanakiri (*n* = 47) provinces, Cambodia

KH group	Province	Mutations in the K13-propeller				Total
		Wildtype	C580Y	R539T	Y493H	
KH1	Pursat	**7**	0	0	2	9
	Ratanakiri	**46**	0	0	0	46
KH2	Pursat	0	**25**	0	1	26
	Ratanakiri	0	**0**	0	0	0
KH3	Pursat	3	7	4	0	14
	Ratanakiri	0	0	0	0	0
KH4	Pursat	0	0	0	**12**	12
	Ratanakiri	0	0	0	**0**	0
KHA	Pursat	15	19	2	6	42
	Ratanakiri	1	0	0	0	1
Total		72	51	6	21	150

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OBJECTIF 3: Etude et définition de la structuration des populations parasitaires circulant au Cambodge pour estimer les zones à risque de diffusion de la résistance à l'artémisinine, en utilisant des approches génomique et bio-informatique.

3.1. Contexte de l'étude

La frontière Khmero-Thaillandaise est connue comme étant le point focal de l'émergence de souches de *P. falciparum* résistantes à la chloroquine, à l'association sulfadoxine-pyriméthamine, à la méfloquine et plus récemment aux dérivés de l'artémisinine (Ariey et al. 2014, Mita et al. 2009, Roper et al. 2004). On estime que la diffusion des souches résistantes à la chloroquine ou à la sulfadoxine-pyriméthamine de l'Asie du Sud-Est en Afrique a été responsable d'une augmentation drastique de la mortalité et de la morbidité du paludisme à *Plasmodium falciparum* (Trape et al. 1993). Il est probable que l'émergence de ces résistances serait due à la structuration particulière des sous-populations parasitaires dans cette région ainsi qu'aux nombreux échanges économiques et mouvements de populations qui existent entre l'ouest et le reste du Cambodge ou la Thaïlande. La fragmentation de la population parasitaire au Cambodge en sous populations, a bien été démontrée en utilisant plus de 800 SNPs caractérisés au sein de 293 isolats par séquençage NGS (Miotto et al. 2013). Les auteurs considèrent que ces sous populations font suite à l'introduction de l'artémisinine. Quatre sous-populations KH1, KH2, KH3 et KH4 et une population mélange KHA ont été identifiées. Les SNPs (single nucleotide polymorphism) sont des mutations ponctuelles sur l'ADN qui varie de façon bi-allélique. D'autres polymorphismes permettent de mesurer et d'étudier la diversité au sein d'une population tels que les variations de nombre de répétition microsatellite, l'apparition d'insertion ou de délétions et les évènements de duplication de gène. Dans ce contexte, nous avons décidé de développer une méthode fondée sur la détection des SNPs, méthode rapide, relativement peu chère, exploitable dans de nombreux laboratoire, permettent d'explorer plusieurs loci.

3.2. Méthodologie expérimentale

La détermination d'un panel de 24 SNPs répartis sur 12 chromosomes (Daniels et al. 2008) nous fournit un point de départ pour définir une technique (appelé « barecode ») pour génotyper les souches de *P. falciparum*. Daniels et al (2008) avait basé leur approche sur la technique TaqMan (real time PCR). Nous avons voulu profiter de l'opportunité que nous avions de disposer d'un appareil de type MAGPIX (Luminex instrument) pour adapter une autre méthode pour détecter les 24 SNPs de Daniels et al (2008). Il s'agit de la technique PCR-LDR-FMA (PCR- ligase detection reaction–fluorescent microsphere based-assay) (McNamara et al. 2006) qui a été développé initialement pour un typage d'espèces *Plasmodium* ou la recherche de SNPs associés à la résistance aux antipaludiques.

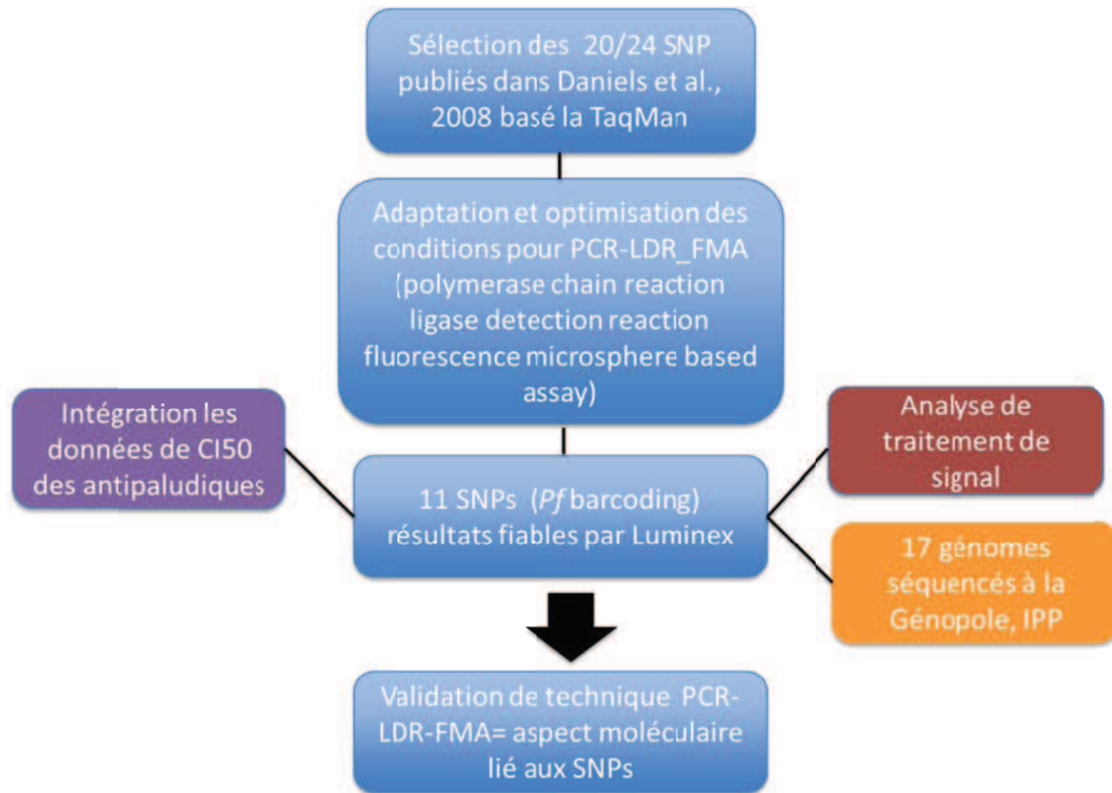

Figure16 : Stratégie de développement de technique PCR-LDR-FMA (polymerase chain reaction ligase detection reaction fluorescence microsphere based assay) à la détection des SNPs.

Dans la phase de validation de la méthode, nous avons validé 11 parmi les 24 SNPs testés.

Nous avons rencontré plusieurs problèmes : (1) la richesse en AT du génome fait que nous n'avons pu dessiner des amorces que pour 20 SNPs parmi les 24 SNPs décrits par Daniels et al. (2008), (2) les signaux enregistrés par MAGPIX n'ont été correct que pour 12 SNPs, (3) un SNP a été rejeté car il était monomorphe sur les isolats testés.

De plus, l'analyse génomique a révélé de nombreux problèmes liés à la structure du génome de *P. falciparum*. Le premier problème était un problème de version du génome. Les loci décrit par Daniels et al (2008) étaient basés sur la version 2 du génome antérieur à la dernière mise à jour de cette version, la version 2.1.5. Il a fallu donc vérifier l'ensemble des séquences en utilisant la version 3 du génome de *P. falciparum*. Nous avons ensuite utilisé la version 11 de l'annotation disponible (PlasmoDB) pour valider chacun des loci. A notre grande surprise, nous avons découvert qu'un des loci décrit par Daniels et al (2008) était dans une région intergénique, de région subtélomérique. Environ un tiers des SNPs décrits par Daniels étaient en position subtélomérique. Les subtélomères de *P. falciparum* sont très grands. Ils couvrent plus de 100 kpb d'ADN à chaque extrémité des molécules. Ces régions sont très variables et codent pour des protéines de surface qui sont souvent organisées en famille multigénique. Le risque est grand en choisissant des SNPs dans ces régions de voir des variations qui ne soient pas spécifique de sous-populations, mais plutôt d'une variation entre individus. Par ailleurs, nous avons vu qu'un des SNPs choisi est localise dans un gène

appartenant à la famille multigénique codant pour les Rifins. Le locus a été rejeté par notre analyse au niveau de la détection LUMINEX. Une des raisons probable de cet échec est certainement due au fait que ce gène est présent de façon quasi-identique sur deux chromosomes différents. L'utilisation des données de génomique apparait comme nécessaire dans le choix des marqueurs moléculaires. Dans la même idée, l'utilisation de la base de données PlasmoDB dans une version antérieure à la version actuelle (v12) nous a permis de voir que le locus BC07 est en fait tri-allélique.

D'autre part, la méthodologie mise en place par Daniels et al (2008) suppose que la fréquence de chaque allèle (MAF, Minor Allele Frequency) soit supérieure à 0.35. Cette valeur est usuelle en génétique des populations. Nous avons voulu vérifier ces valeurs en comparant les données obtenues par Daniels sur un échantillon restreint d'individu du Sénégal et de Thaïlande, avec plus d'échantillons disponibles dans la base de données MalariaGEN. La version 1 de cette base de données était bâtie sur 825 génomes séquencés dont 293 au Cambodge. En 2014, une version 2 bêta a été mise en ligne. Elle repose sur plus de 5000 séquences réparties dans le monde entier. On a pu remarquer que les fréquences alléliques variaient énormément en fonction de zones considérées. Plus surprenant, nous avons constaté un changement de fréquence significative en augmentant la résolution géographique. Nous avons été confrontés à ce problème dans le cas de notre analyse. Plusieurs des 11 SNPs validés pour l'analyse finale sont avérés non valide suivant le critère initial de MAF > 0.35. Egalement, suivant nos analyses statistiques, il s'est avéré que le critère MAF >0.35 n'est pas forcément un critère pertinent pour notre étude.

3.3 Reconstruction du génotype

Pour cela, nous avons développé une approche intégrée prenant en compte la mesure brute obtenue à partir de l'appareil utilisé pour fournir un génotype complet pour les 11 SNPs sélectionnés. La première difficulté a été liée à l'hétérogénéité du signal généré par la méthode choisie. Le principe de la technique LUMINEX est d'utiliser des billes de polystyrène microscopiques (microsphères) couplées à des molécules de détection spécifiques. La technologie xMAP couplée à l'appareil de détection MAGPIX utilise les microsphères en polystyrène aimantée. Dans le cas des hybridations ADN-ADN, les molécules spécifiques sont des amorces permettant de détecter un allèle donné. Dans le cas de la technique PCR-LDR-FMA, le complexe d'hybridation est couplé à la biotine et est détecté par le conjugué Streptavidine-Phycoérythrine (SAPE : Streptavidin, R-Phycoerythrin Conjugate). L'appareil utilisé possède deux LED, une LED rouge (635 nm) qui identifie les billes étudiées et une LED verte (535 nm) qui détecte le signal de fluorescence liée au couplage biotine-streptavidine. Ainsi, pour chaque SNP nous avons obtenu deux valeurs lumineuses correspondant aux deux allèles avec des signaux de deux billes différentes. Le problème que nous avons dû résoudre était que le signal obtenu pour chaque allèle était extrêmement différent.

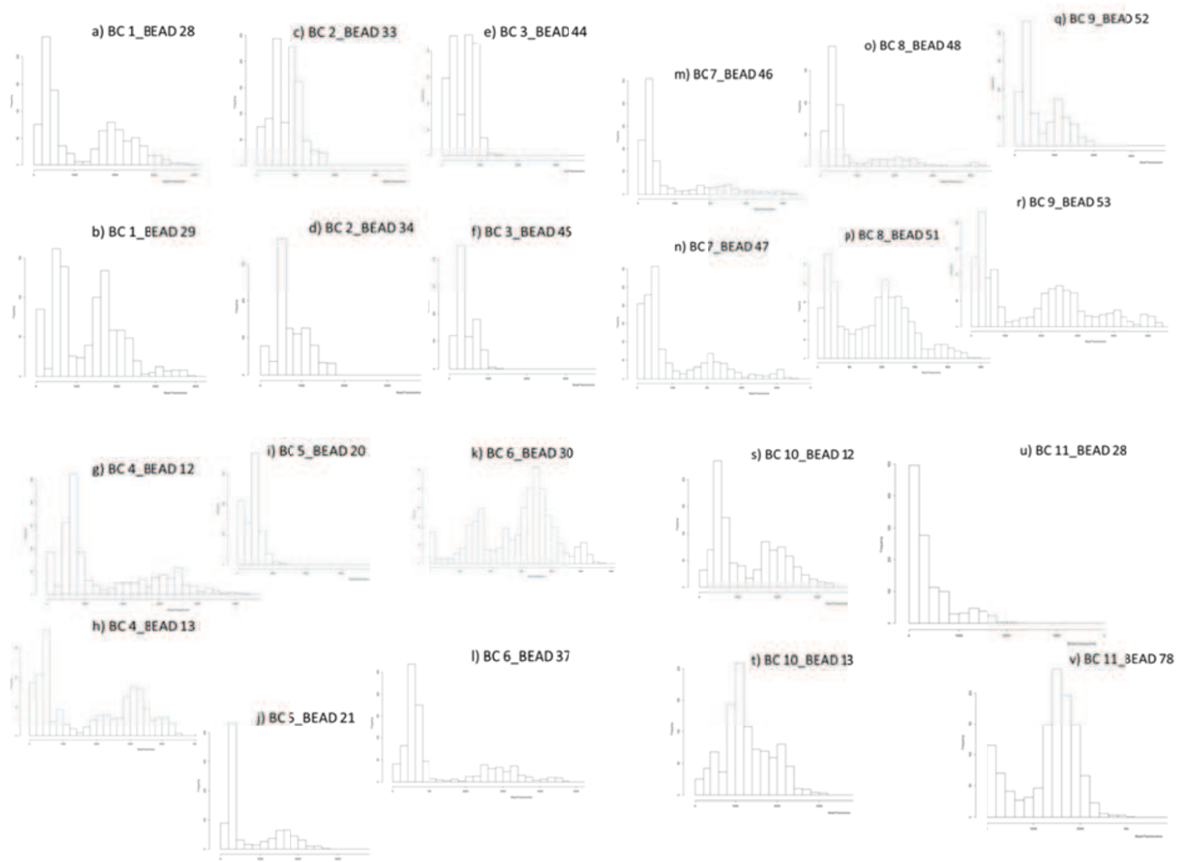

Figure 17 : Hétérogénéité très importante des mesures de fluorescence en fonction des billes et des amorces utilisée pour le test PCR-LDR-FMA (chaque histogramme est dessinée à partir de 500 à 700 mesures : l'axe x correspondent aux valeurs d'intensités du signal fluorescent émit, l'axe y correspondent aux effectifs par classe).

Nous avons constaté que la technique LUMINEX fourni un signal lumineux qui varie en fonction des billes utilisées ayant aussi bien pour le signal que pour le bruit de fond (Figure 17). Nous avons supposé que les valeurs suivent une distribution centrée autour d'une valeur moyenne aussi bien dans le cas d'un signal positif (signal) que d'un signal négatif (bruit de fond). Dans ce travail, nous avons développé un algorithme nous permettant de classer les mesures effectuées en fonction des deux populations signal et bruit de fond. L'algorithme a été fondé sur une minimisation des variances entre les deux mesures. Une mesure a ainsi été considérée comme positive, négative ou non interprétable si nous n'arrivons pas à exclure la mesure d'au moins une des deux populations. Nous avons voulu travailler par plaque 96 puits car nous ne maitrisons pas la variation inter-plaque dans cette expérience.

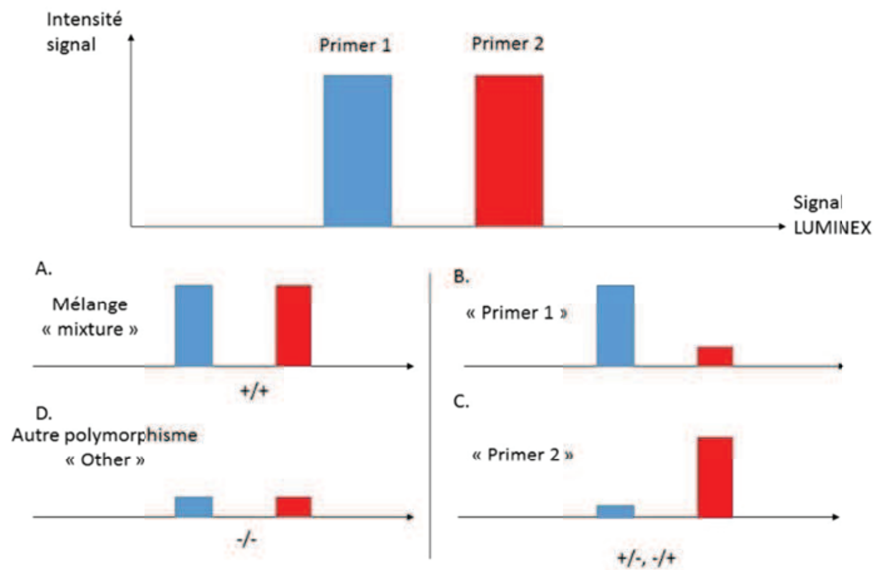

Figure 18 : Schéma de décision permettant de déterminer la valeur d'un allèle.

La valeur d'un SNP pour un isolat basée a été déduite en fonction des deux mesures correspondant aux deux allèles. Quatre situations étaient possibles (Figure 18) dans le cas où les mesures pour les deux allèles étaient valides. A un SNP donnés, la décision peut être Primer 1 (allèle 1), Primer 2 (allèle 2), Mixture (infection polyclonale), ou Autre (ni l'allèle de référence ni l'allèle testé avec le deuxième primer). Dans 99% des cas, nous avons validé les données fournis par l'algorithme (Tableau 1). Pour valider notre approche nous avons utilisés le 17 génomes qui ont été séquencés à la Génopôle, Institut Pasteur Paris.

Primer 1	**Primer 2**	**Décision**
Positif	Négatif	Primer1
Négatif	Positif	Primer2
Positif	Positif	Mixture
Négatif	Négatif	Autre
Positif	Non interprétable	Primer1
Non interprétable	Positif	Primer2
Négatif	Non interprétable	Non Valide
Non interprétable	Négatif	Non Valide
Non interprétable	Non interprétable	Non Valide

Tableau 1. Arbre de décision pour la valeur d'un locus pour un individu donné.

3.4 Echantillonnage

Nous avons échantillonné 418 prélèvements sanguins collectés de patients infectés par *Plasmodium falciparum*, entre 2010 et 2011, dans 15 centres de santé répartis dans 10 provinces du Cambodge (Figure 19). Nous avons pu rassembler des données provenant de toutes les régions où la transmission du parasite est active. Seul 221 échantillons ont pu être complètement caractérisés. Ce taux de réussite d'un peu plus de 50% cache en fait de grande différence territorial. Nous avons remarqué que certains centres de santé présentaient un taux de plus de 50% d'infection polyclonale. Nous avons également observé sur un grand d'échantillon qu'un allèle au moins était mal déterminé. C'est une des limites de la méthode qui repose sur 22 expériences différentes avant de pouvoir reconstruire le génotype.

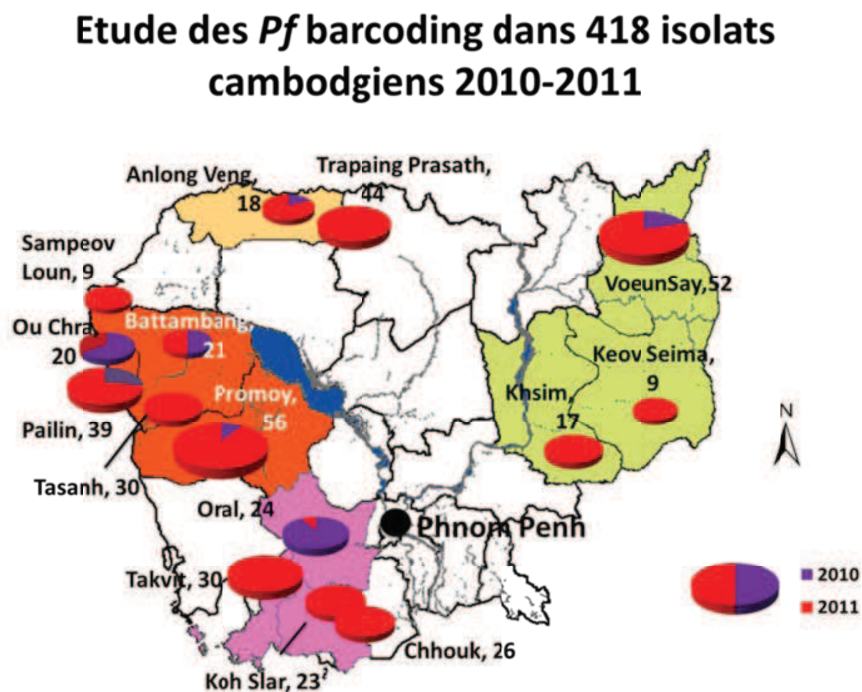

Figure 19 : Echantillonnage de l'étude Pf barcoding dans 418 isolats cambodgiens entre 2010 et 2011.

3.5. Analyse et Résultats

Nous avons choisi de prendre plusieurs indicateurs dans cette étude pour évaluer les flux des gènes au sein de la population parasitaire au Cambodge.

Le premier critère a été la fréquence allélique. Nous avons vu que la fréquence d'un allèle pouvait varier énormément en fonction de la résolution géographique choisie. Grâce à l'échantillonnage de l'étude, nous disposions d'une couverture fine du territoire. Nous avons constaté que des différences importantes

apparaissent dans certaines régions. Nous nous sommes donc intéressés aux allèles qui présentaient une différence significative par rapport aux autres allèles (déséquilibre important entre les fréquences des allèles), ce qui nous permis de faire apparaitre les différentes sous populations ainsi que les flux d'allèle entre les régions. On remarque que dans certains cas, la résolution géographique était juste suffisante, comme par exemple à l'ouest du Cambodge entre la région de Battambang et celle de Pailin ou au sud entre les centres de santé de Takvit et de Koh Slar. Le choix de travailler par centre de santé nous a imposé aussi certaine limitation à notre étude. Il serait plus juste de travailler avec l'origine géographique des patients à l'avenir (mais si ce type d'information est très difficile à obtenir en routine). Nous avons également observé (à Pailin, mais surtout dans le nord à Anlong Veng), une perte de signal ou tout au moins un mélange du signal avec les échantillons obtenus via les centres hospitaliers qui couvrent de plus grands territoires (et drainent plus de patients) que les centres de santé. Nous avons mesuré un indicateur de redondance mesurant, pour une région donnée, le nombre de fois où un génotype était répété. La redondance au niveau du génotype suggère que des individus très proches ont infectés des patients différents. Cet indicateur vient en complément de la valeur de Fst que nous avons calculée pour chaque individu puis de façon moyenne par centre de santé.

Enfin, nous avons essayé d'identifier des individus pouvant être regroupé sur la base de leur génotype. Nous avons donc comparé différentes approches de clustering pour ne garder que les individus restant associés quel que soit la méthode employée. Les clusters obtenus nous a permis de confirmer les informations fournies par les autres marqueurs. Cependant, aucune association significative n'a été mise en évidence entre les 11 SNPs et les tests de chimiorésistances basés sur la mesure de la CI50% (concentration inhibitrice d'antipaludiques nécessaires pour inhiber le développement de 50% des parasites). Nous avons juste confirmé des différences significatives entre les moyennes géométriques des CI50 pour la chloroquine, la méfloquine, l'artésunate et la dihydroartémisinine des populations parasitaires circulant à l'Ouest, à l'Est, au Nord et au Sud du Cambodge. Aucune liaison génétique n'a pu être établie entre les allèles utilisés pour le barecode et les allèles mutés présents dans le domaine propeller du gène Kelch 13 (K13, PF3D7_1343700), récemment impliqués dans la résistance à l'artémisinine.

En conclusion, nous avons bien retrouvé les sous-populations décrites au Cambodge comme jouant un rôle important dans la propagation de certains allèles. Nous avons également confirmé l'existence des populations mélange, populations avec une distribution géographique plus large et suspecter de jouer un rôle essentiel dans la transmission.

3.6. Perspectives

La technique Luminex a été correctement mis en place au laboratoire. Nous souhaitons donc augmenter le nombre d'isolats analysés. Pour cela, nous disposons d'une collection de plus de 600 échantillons. De plus, notre analyse nous a permis de constater l'importance de maitriser l'analyse du signal généré expérimentalement avant de procéder à l'analyse statistique. Nous avons en effet remarqué que les allèles ne portent pas tous le même niveau d'information : les allèles BC01, BC06 et BC11 se sont révélés très peu utile, quel que soit l'indicateur utilisé. De ce fait, il sera plus facile dans l'avenir d'appliquer l'algorithme mis en place à d'autres génotypages ou bien encore à la caractérisation de jeux d'allèles comme les allèles Kelch impliqués dans la résistance à l'artémisinine.

Les premiers résultats montrent que les flux parasitaires ont lieu dans de nombreuses directions. La présence de différentes sous-populations est clairement établie dans toutes les régions du Cambodge, ce qui suggère que la fragmentation de la population parasitaire est plus importante que celle décrite par (Miotto et al. 2013). La présence de populations mélanges s'est avérée être un indicateur intéressant, en particulier en raison de la très forte prévalence d'infection polyclonale dans certaines régions comme la région d'Oral. Malheureusement, notre étude a été limitée par le choix des SNPs initialement choisi. Aucun de ces SNPs ne s'est révélé fortement aux allèles K13. Les génotypes obtenus clustérisaient très mal. Ceci suggère que les allèles étudiés sont peu représentatifs des sous-populations présentes actuellement au Cambodge et que nous devons maintenant définir des nouveaux allèles très spécifiques de chacune des sous-populations KH1, KH2, KH3 ou KH4 déjà décrite par (Miotto et al. 2013) ainsi que des sous-populations mélanges telle que KHA. Il nous faut pour cela intégrer des données de génomique absentes des banques de données telles que PlasmoDB ou MalariaGEN. Dans le cas de MalariaGEN, les données de SNP par isolat sont privées. Néanmoins, il possible d'envisager de récupérer les données de séquences déposées dans la base de données ENA (European Nucleotide Archive). Les données NGS concernant les 293 génomes séquencés par la Wellcome trust (Miotto et al. 2013) sont présentes dans des formats variés. Nous avons identifiés près de 200 fichiers qui sont au format .bam, qui correspond aux read alignées sur le génome de référence. Nous pouvons envisager une extraction des variants pour utiliser plus de SNP et ainsi retrouver les sous-populations déjà décrite. Cette approche est fastidieuse. Elle montre cependant que le choix de ne travailler que sur les valeurs de MAF n'est pas suffisant pour mener une analyse de population. On peut espérer que la base de données MalariaGEN évolue favorablement.

En complément de ma thèse, j'ai également pu séquencer 17 génomes de *P. falciparum* à l'occasion d'un stage sur la plate-forme de génomique PF1 de la Génopôle de l'Institut Pasteur à Paris. Les 17 échantillons d'ADN avec les phénotypes connus (valeur RSA) venant de Pailin, Pursat (à l'Ouest), Oddar Meanchey (au Nord), Rattanakiri et Kratie (à l'Est) en 2010 et 2011 ont été entièrement séquencés par la technologie Illumina en paired-end (Ariey et al. 2014). La technique Illumina fait partie des nouvelles technologies de séquençage (Next Generation Sequencing ou NGS). Nous avons obtenu de très nombreuses séquences nucléotidiques courtes (« short reads ») qui ont été compilés dans un fichier type Fastq. La

couverture moyenne des reads a été de 300-400x, c'est à dire qu'à une position donnée sur le génome, nous avons obtenu 300 à 400 fois une information nucléotidique. Les séquences Fastq ont ensuite été traitées pour éliminer les séquences de mauvaise qualité (souvent à l'extrémité). Les séquences ont été alignées avec la version 3 du génome de 3D7 afin d'extraire les SNPs. Seul les SNPS présents dans la partie des CDS ont été conservés dans une première analyse.

Nous espérons pouvoir ainsi déterminer, à travers ces approches de séquençage NGS, les SNPs, génotype et haplotypes des principales sous-populations de parasites présents au Cambodge. Il apparait évident à travers cette étude que la fragmentation de la population parasitaire a été un obstacle majeur à toute les approches mise en place pour identifier le gène impliqué dans l'acquisition de la résistance à l'artémisinine. Ce gène a pu être trouvé grâce à une approche originale décrite dans l'objectif 2 de la thèse. Nous disposons maintenant de plus de 50 génomes séquencés à l'Institut Pasteur à Paris. Un de mes souhaits serait donc poursuivre mon travail sur l'analyse fonctionnelle des sous-populations cambodgienne de façon à mieux comprendre leur origine.

Manuscrit en préparation 2

Plasmodium falciparum parasite population structure and population gene flows associated to antimalarial drugs resistance spreading in Cambodia

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INTRODUCTION

Malaria is one of the most severe global health concerns in the world and the emergence and the spread of multidrug resistant parasites are the main challenge we are facing, at present. The Cambodian-Thai border is recognized as the epicenter of the emerging resistances. P. *falciparum* clinical malaria resistance to chloroquine has been first documented in 1957 (Eyles et al. 1963, Harinasuta et al. 1965) in this area. Later on in 1967 pyrimethamine resistance were also reported in the same region (Peters 1987, Wongsrichanalai et al. 2002). Molecular epidemiological studies have confirmed that the spread of resistant parasites to these two drugs to Africa has originated from Southeast Asia (Mita et al. 2009). In 1990s, mefloquine resistance has been consequently observed in this area and more recently, the emergence of artemisinin derivatives was established along Cambodian-Thai border (Dondorp et al 2009 Noedl et al. 2008). The reasons of the emergence of multi resistance parasites in this area remain unknown. Recently, whole genome sequencing data (Miotto et al. 2013) demonstrated that *P. falciparum* populations were highly fragmented in Cambodia. Four subpopulations (KH1, KH2, KH3 and KH4) and one large admixed population (KHA) were described. The KH2, KH3 and KH4 subpopulation were associated to artemisinin resistance defined by a delayed of the parasite clearance in the first three days of artesunate monotherapy treatment (Miotto et al. 2013) and confirmed later to be associated to mutations in the Kelch 13 propeller domain (PF3D7_1343700) (Ariey et al. 2014). In their report, Ariey and collaborators clearly shown that the prevalence of mutant alleles in the Kelch-propeller domain, involved in artemisinin resistance, was much higher in Western provinces than in Eastern provinces(Ariey et al. 2014). One hypothesis is the structure of the parasite population plays an important role in the spread of K13 mutant alleles from West to East Cambodia. The structure of the parasite population can be assessed using the study of different genetic markers such as the single nucleotide polymorphisms (SNPs), the microsatellite repeats, the insertions/deletions and the range of gene duplication events. Several molecular approaches have been developed to detect accurately and reliably the SNPs in the *P. falciparum* genome. For instance, a 24 SNPs set was recently described by Daniels and collaborators (Daniels et al. 2008) detected by a robust TaqMan genotyping approach. Their analysis was performed on African and Thai isolates. At present, novel, rapid and reliable technics based on fluorescent magnetic beads, such as the LUMINEX technology, have been developed to detect specific alleles. A rapid assay of *Plasmodium* typing was developed using fluorescent microspheres (McNamara et al. 2006). This assay combined a PCR and a ligation reaction: PCR-LDR-FMA for PCR-based ligase detection reaction–fluorescent microsphere assay.

In the present study, we describe the development and the implementation of the PCR-LDR-FMA applied to the detection of 11 SNPs. for a barcode approach based. The barcode detection method described here, integrates a specific signal analysis and genotype reconstruction algorithm. By using this method, we also evaluated the presence of parasites subpopulations and intensive gene flow overt the country that could support the spread of drug resistance.

MATERIALS AND METHODS

Samples size

We analyzed 418 blood samples collected in 2010-2011 from *falciparum* malaria patients coming from 15 health centers (11 health centers and 4 reference hospitals) located in 10 provinces in Cambodia Samples were stored at -20^{0}C (Tab. S1). Isolates were grouped in four major regions: West, South, East and North Cambodia. .

DNA extraction and amplification

The genomic DNA was extracted from 200 µl of blood by using a DNA blood kit (Catalog 51306, Qiagen, Germany) according to manufacturer's instructions. DNA extracts were stored at -20°C until use. DNA from reference strains 3D7, Dd2, HB3 and Ro33, provided by Malaria Research and Reference Reagent Resource Center-MR4, were used as controls. Primary PCR was carried in 25 µL of final volume with 0.25 µM of each corresponding primers, 0.2 mM of each deoxynucleoside triphosphate (dNTP) (Solis Biodyne), 1x of reaction Buffer, 2.5 mM of MgCl2, 1.25 U FirePol® Taq DNA Polymerase (Solis Biodyne). The primary PCR was done with the conditions: 94°C for 15 min, then 30 cycles of 94°C for 30 sec, 52°C-55°C for 1 min (Tab. 1), and 72^{0}C for 1 min and final extension at 72°C for 10 min to reach the corresponding target between 164 bp and 385 bp.

The Nested PCR was performed in 25 µl of final volume in 0.5 µM of each primer, 0.2 mM of each deoxynucleoside triphosphate (dNTP) (Solis Biodyne), 1x of reaction Buffer, 2.5 mM of $MgCl_2$, 2.5U Taq polymerase (FirePol® DNA Polymerase, Solis Biodyne). We used 5 µl of the primary PCR reaction as template. PCR conditions were: 94°C for 15 min, then 40 cycles of 94°C for 30 sec, annealing temperature between 55°C-60°C for 1 min and 72°C for 1min. A final extension at 72°C for 10 min was performed to obtain the corresponding fragments between 100 and 200bp). PCR of valid SNPs were performed in four multiplexed reactions (Tab. 1).

Ligation and detection assays

Nested PCR products were pooled together in two sets according to microsphere combinations.one µL of the pooled PCR products was used for the Ligase detection reaction (LDR). The LDR was based on 2 allele-specific primers and 1 locus- specific probes (Tab. S3). For each reaction, a large LDR primer and a small primer were used (Fig. 1). The allele-specific primers were composed of two parts: the 5-prime part hybridizing with the MagPlex-Tag probe and the 3-primer part hybridizing with the PCR product. We used 33 different MagPlex-Tags to detect 40 alleles (Tab. S3) corresponding to the 20 loci which were successfully amplified by PCR (Tab. 1). Ligation was performed after hybridization of the locus-specific primer. Several MagPlex anti-Tag probes were used twice. Locus-specific probes were 5' phosphorylated and 3'biotinylated. LDRs were performed in a final volume of 15 µL holding in 1X of Taq Ligase buffer, 10 nM of each LDR (allele- and locus-specific primers), 4 U of Taq DNA ligase (Genesearch) and 1 µL of pooled Nested PCR. Thermocycling conditions were carried out by a denaturation of the double strand DNA at 95°C for one minute, followed by 32 cycles at 95°C for 15 seconds and hybridization at 58.0°C-60^{0}C (Tab. S3) for 2 minutes. We performed the control quality by using DNA from reference strains were provided by MR4. Two multiplexed reactions were used to characterize final valid SNPs (Tab. 1)

Hybridization and labeling of magnetic beads

A 5 µL fraction of the LDR product was poured into 60 µL of hybridization solution TMAC buffer (3x of tetramethylammonium chloride [TMAC] (Sigma-Aldrich), 3 mM of EDTA (Gibco), 50 mM Tris-HCl, pH 8.0 (Sigma-Aldrich), 0.1% sodium dodecyl sulfate) and 1000 beads of each MagPlex-Tag microspheres used in the multiplex LDR described above. We performed the bead quantification as previously described (Bruse et al. 2008). Mixtures were heated to 95°C for 1 min 30 sec and incubated at 37°C for 35 minutes to allow hybridization between SNPs-specific LDR products (Tag-probe) and bead-labeled anti-TAG probes (Fig. 1). Then, 6 µL of 1:50 dilution of streptavidin-R-phycoerythrin (Invitrogen) in TMAC buffer was added to the post-LDR mixture and incubated at 37°C for 20 minutes in 96-well plate (Eppendorf). PCR and LDR reaction were conducted in 96-well plate. The fluorescence of each allele-specific LDR products was measured on a MagPix instrument with xPonent 4.2 software (LUMINEX).

Signal detection and analysis

We first characterized the measurement of the signal *m* for a primer *k* and for a series of sample $E_k(i)$, $1 \leq k \leq 2$. A signal *m* can be decomposed into two components:

$$m = M + \varepsilon,$$

where M is the signal intensity without noise and ε is the background noise. A series of samples E_k can be thought of as a distribution between two data sets: a first group composed of samples $E_k(-)$ of noised signal and an opposite group $E_k(+)$ composed of samples where the noise represent a low proportion of the signal.

We have $E_k = E_k(-) \cup E_k(+)$, with $E_k(-) \cap E_k(+) = \emptyset$.

Therefore, the signal m can be further decomposed as:

1) $m = \varepsilon$, ($M = 0$) or 2) $m = M + \varepsilon$, ($\varepsilon << M$).

Identification of $E_k(-)$ and $E_k(+)$sets is based on a classification method minimizing the variance associated to the two series of measures. This algorithm is analogous to the k-mean algorithm where k = 2.

1) It begins by an initiation phase where the minimum and maximum values are selected.

seed1 $\leftarrow$ *max (m_pr*$_k$*(i))*, *seed2* $\leftarrow$ *min (m_pr*$_k$*(i)),*

2) The two series are created

$E_k(+) = \{seed1\}$, $E_k(-) = \{seed2\}$,

3) *Variance* $\leftarrow$ *Variance* $(E_k(i))$

4) *Var(+)* $\leftarrow$ *Variance/2, Var(-)* $\leftarrow$ *Variance/2, cpt* $\leftarrow$ *0*

5) The growth of the two sets is obtained by minimizing the sum of their variances. The center of gravity of each data sets is calculated for the reattribution of each values to the two sets by minimizing the variances.

```
Repeat
        Variance ← Var(+)+Var(-)
        cpt ← cpt+1
        For i=1 to n
                If Abs(germe1 – m_prk(i)) > Abs(germe2 – m_prk(i)) then
                        Ek(+) ← Ek(+) U {m_prk(i)}
                If not
                        Ek(-) ← Ek(-) U {m_prk(i)}
                End if
        Next i
        seed1 ← barycenter(Ek(+)),
        seed2 ← barycenter(Ek(-))
        Var(+) ← variance(Ek(+))
        Var(-) ← variance(Ek(-))
```

Until cpt>20 and variance<var(+)+var(-)

A test was used to address each measurement to the negative or positive value of the allele. We assume that both series of values $E_k(-)$ and $E_k(+)$ are following a Gaussian distribution (Fig. 2e). For each primer k, we consider $M\mu_k$ and $M\sigma_k$ as the mean and standard deviation associated to the distributed of the signal without noise. Similarly, $\varepsilon\mu_k$ and $\varepsilon\sigma_k$ are the mean and standard deviation associated to the distributed of the noised signal. The signal m is positive if it is significantly higher than the noise. On the opposite a signal is considered to be negative if it is significantly less than the signal without noise. The random variable X corresponds to one measurement of a series E_k. We calculate the probability $P1$ and $P2$ to reject the null hypothesis $H0$ whilst $H0$ is true for the distribution of the signal without noise ("signal") and for the noised signal ("noise") respectively.

$P1 = P(X>m/ H0_1, X \text{ has } N(M\mu_k, M\sigma_k))$, for the signal

$P2 = P(X<m/H0_2, X \text{ has } N(\varepsilon\mu_k, \varepsilon\sigma_k))$, for the noise.

The overall hypothesis test can be expressed as $H0: H0_1$ *or* $H0_2$ (exclusive), $H1$: *No* $H0_1$ *and No* $H0_2$. The decision rules become:

If $P2 < P1$ then m is a negative measure for the presence of the allele

If $P1<P2$ then m is a positive measure for the presence of the allele.

It is one exception if $P1>1-0.0027/2$ *and* $P2>1-0.0027/2$, then the measure is considered as uncertain. The final decision concerning uncertainties could be reconsidered manually.

Decisions resulting from the algorithm were validated on each 96-well plates using for reference DNA for sequenced strains: 3D7, Dd2, HB3 and RO33. The overall genotyping approach was validated on the genome sequence of 17 Cambodian isolates (data not shown).

Data and Statistical analysis

Statistical performed using R software (http://www.r-project.org/). Allele frequency observed in the different health centers over Cambodia was evaluated using Chi-square tests for independence. Loci presenting a *p-value* < 0.05 were considered as significant. We highlight all Chi-square components values which were over 2 (Tab. S1). The distribution of drug sensitivity among geographic areas and between alleles was performed using ANOVA test. Genetic linkage between the 11 SNPs tests and comparison with Kelch 13alleles was performed using Tschuprov distance. Redundant and non-redundant sets of genotypes were compared by constructing first a distance matrix based on pairwise distance or *Fst* parameter and using afterward Ward clustering approach. A threshold was applied to identify 9 clusters in each classification. The clustering of isolates with four different approaches was recoded to enable classification. Only isolates that

cluster always together was further analyzed. Genotypes were aligned and a consensus sequence was drawn using weblogo (http://weblogo.berkeley.edu/logo.cgi).

RESULTS

Selection of the Single Nucleotide Polymorphisms

A total of 418 samples collected between 2010 and 2011 were tested (Tab. S1). A probe for each of the 24 SNPs previously described (Daniels et al. 2008) was designed . Among the 20 independently PCR fragment corresponding to the 20 SNPs successfully amplified only 13 assays were interpretable (Tab. 1). The assay #7 was abandoned due to non-reproducibility and non-accuracy of the detection on control DNAs. Assay #5 was rejected because genotyping analysis revealed that this locus was monomorphic. Finally, 11 SNPs were validated. Four multiplex PCRs and two multiplex LDRs were optimized for increasing the LUMINEX results according to their annealing temperature.

All probe sequences were designed using the version 3 of *P. falciparum* genome. We observed that 11 SNPs amongst the 24 described by Daniels *et al.* (Daniels et al. 2008) were located in a coding region (synonymous mutations) (Tab. 2). One locus was located in an intergenic region while the other SNPs corresponding to non-synonymous mutations. Five SNPs were located in subtelomeric regions. Genomic analysis revealed that negative detection of assay #11 is due to the presence of two nearly identical copies of the Rifin gene in which the SNP is located (Tab. 2). PlasmoDB v11.1 suggested that assay #15 corresponding locus is indeed tri-allelic, thus explaining the high rate of negative results in our data. Strong discrepancy are observed over the world between *P. falciparum* population emphasizing the huge difficulty to evaluate from a single analysis the quality of SNPs for population studies Assay #24 was validated for LUMINEX genotyping despite its low expected variation in Cambodian parasite population (Tab. 2).

Barcoding.

The heterogeneity of the signal measured by the MAGPIX machine was high (Fig. 2). We performed an analysis of the value of a SNP for an isolate in two steps: 1) the LUMINEX signal was analyzed for each allele and 2) the resulting conclusions were combined in a decision tree for the two alleles (Tab. 3).

For the first step, we developed an algorithm that classifies set of signal values characterizing the presence of one allele at a specific locus. The classification of a signal values between positive signal and noise background was performed using a method minimizing the

variance associated to the two series of measures which is analogous to the k-mean algorithm where k = 2. At step two, the decision concerning allele instance could be considered as reference or altered according to 3D7 reference genome, mixture corresponds to polyclonal samples, "Other" suggesting that we were not able to characterized the allele value at the corresponding locus based on the two primers used in the present analysis, or "Not valid" which corresponds to experiments that either should be confirmed or to DNA samples of bad quality. We successfully genotyped 221 samples with 11 SNPs among the 418 samples.

Allele frequency

Frequencies observed in the 221 samples were close to MalariaGEN reports except for BC06 was 2 fold higher than expected (Tab. 2). Discrepancy may result from the sampling. Our analysis was restricted to malaria transmission areas in Cambodia. Very few alleles were restricted to a single region but frequency varied drastically, suggesting a flux between the different areas in the country. Each SNP distribution was compared over the sampling sites (Fig. 3). SNPs BC01, BC06 and BC11 were equally distributed over the country. The Chi-square analysis shows that the west part of Cambodia, including Battambang, Pailin and Pursat provinces displayed the more significant SNPs. We observed that for most of the loci, significant Chi-square differences from equal distribution of both alleles (BC02, BC03, BC04 and BC09). We focused our analysis on loci presenting a strong local disequilibrium to emphasize the presence of parasites flow and subpopulations. We found several alleles present at significantly low frequency in the North Cambodia (minor alleles: BC02_ALT, BC04_REF, BC05_ALT, BC09_REF, $p < 0.05$). North Cambodia might contain specific subpopulations. Input of alleles from the South and the West part of the country also exist. The BC03 alternative allele was absent in Ratanakiri (Veurn Say Health center) suggesting the presence of a specific subpopulations in this region. We also observed in Chhouk, in South Cambodia the quasi-absence of alleles BC02_ALT and BC04_REF (Tab. S1, see major allele distribution, Fig. 3).

Drug resistance

We used IC50s *in vitro* drug susceptibility to evaluate the level of significance of the 11 SNPs regarding spreading of drug resistance in Cambodia. Drug resistance was performed *in vitro* routinely when parasitemia was over 0.1%, as described previously (Leang et al, 2012). The geometric mean IC50s of isolates from the Eastern Cambodia was lower than in the rest of the country whatever the drug considered (Fig. 4). Difference was significant for for artesunate (ART, p<0.01), dihydroartemisinin (DHA, p<0.1) and mefloquine (MFL, p<0.05). Sample from

Battambang hospital and Oral showed highest IC50 values for ART ($p<0.001$). DHA IC50s were significantly higher in Battambang. ART and DHA IC50s correlated to each other but showed no correlation with any of other drugs. Mefloquine IC50s were significantly higher at Takvit health center than in the other health center from the South part of the country ($p<0.001$). No association between IC50 s and our panel of SNPs was observed. We successfully amplified and sequenced the K13 propeller domain in the PF3D7_1343700 gene (Ariey et al. 2014) for 70 isolates. We confirmed that alternatives alleles were mostly associated to the West and the North Cambodia ($p<0.01$). The C580Y allele was the most prevalent (59/70). The R539T was the second most frequent allele (9 isolates). Four of these R539T isolates were from the north Cambodia. No association between K13 mutations and barcode SNPs was found. We also failed to identify linkage with artemisinin resistance suggesting that several subpopulations may support alleles involved in drug resistance.

Population genetics

We used several approaches to confirm that biased alleles frequency described above results from the existence of subpopulations and genetic exchange in Cambodia. Correspondence analysis using SNP data showed that some alleles had a remarkable distribution over health centers and major sectors where malaria is endemic (North, South, East and West) (Fig. 5). The BC02 altered allele was strongly associated to Western Cambodia. On the opposite, the BC05 alternative allele was associated to Eastern Cambodia. These two alleles suggest that exchange between Eastern and Western populations exist in both direction. The situation was the same between Western and Northern Cambodia according to BC07 loci. The altered allele G was associated to health centers located in the West whereas the altered allele A was strongly associated to Anlong Veng and Trapaing Prasat health centers. The health center of Oral was not associated to any cardinal points. The presence of a high level of admixed parasite population in that region suggest that it is subjected to various parasite input. Results were in agreement with previous assumption that strong economic exchange existing along that South-West axis. The number of valid samples was low in that region, but was hampered by the present of a large amount of polyclonal infection (Tab. S1).

Flows between subpopulation were illustrated by local analysis of significant biased in allele frequency (Fig. 3). Pailin appears as the recipient of several populations flow. The BC02 altered allele was the only one found in Tasanh health center samples for BC02 locus (Tab. S1). The frequency of this allele was higher than expected in Pailin (Ou Chra health center and Pailin hospital, Tab. S1). The BC02 alleles were equally distributed at Battambang hospital (no significant distribution bias, Fig. 3), suggesting a flow between Northern and Southern Pailin, the region of Tasanh being donor for altered allele. The process was also illustrated by the two altered allele of

locus BC07. The altered allele G was nearly the unique allele in both Tasanh health center and Battambang hospital (Tab. S1) and its frequency was considerably high in Pailin (11/2/4 and 14/7/6 for Ou Chra health center and Pailin hospital respectively, Tab. S1). The overall allele diversity was higher for BC07 locus in Pailin. The origin of the BC07 altered allele A could either be from the South, according to Promoy health center or from the north where the A allele was the major allele (Anlong Veng hospital and Trapaing Prasat health center, Tab. S1). The dynamic of allele distribution was differently supported in the North West part of the country (31%) were the average Fst distribution was remarkably low compare to the South East part of the Country (45%, Fig. 6). This was confirmed by the level of redundancy of barcodes which was much higher in samples from the North and the West. The redundancy level was 1 – (Nb of different genotypes / Nb of samples). We observed 165 different genotypes among the 221 samples. The global redundancy level was 0.25. It was much higher in the North Cambodia (approx. 0.5) and decreased progressively to the South (Pailin: 0.3, Pursat: 0.4, Kampong: 0.33, Chhouk: 0.19). This level was only 0.1 in Ratanakiri and 0.33 in Kratie. This region was also the barycenter of several genotypes representing stable groups of parasites described in the parasite population using various clustering methods (Group 1, 2 and 4, Fig. 7). By varying the clustering approaches (based either on pairwise distance matrix, the other one using Fst as distance) and the number of samples (using either redundant or non-redundant data), we failed to obtained large stable clusters. Nevertheless we found five stable groups of isolates that focus our attention (Fig. 7). Group 3, 4 and 5 were strongly associated to C580Y K13 mutant allele. Group 5 contained 12 samples from Pailin and 3 from Battambang. Group 3 isolates were mostly distributed in the South.

DISCUSSION

We have optimized a PCR-LDR-FMA technique for the high throughput detection of *P. falciparum* barcode for Cambodian isolates. This strategy included multiplex PCR and ligase detection reactions prior to hybridization with magnetic microspheres (MagPlex-Tag probes). The signal discrimination between the 22 alleles corresponding to 11 SNPs was based on a specific algorithm working in three different steps. The different steps were fully automated In the future; the implementation of a dashboard for data management along the process would greatly improve genotyping approaches based on microsphere assays.

The choice of the loci is a critical step for barcode analysis. It was based on both allele frequency and possibility to run a PCR reaction at the corresponding locus. Little information was available regarding the allele frequency in Cambodia at the beginning of the study. We used validated SNPs described by Daniels et al., (Daniels et al. 2008). The frequency in our dataset (Tab.

S1) was sometime below the expected major allele frequency (MAF) although Daniels et al study included Thai samples. The MalariaGEN database revealed the dramatic variation of MAF over the world including selected SNPs. Moreover, the genomic analysis revealed that some SNPs were chosen within genes that are associated to the subtelomeric regions and/or that encode surface antigens (Tab. 2). These two features are generally associated with highly variable regions of the genome. In that case, MAF value will not be relevant for population studies. The PCR-LDR-FMA required the design of several primers at each locus. This task was hampered by the AT richness of *P. falciparum* genome. This was the main source of rejection of candidates. Despite the development of databases it remains difficult or impossible to find alternative locus when the probe design failed at a first genome location. The development of future databases on co-occurrence of markers in populations and haplotypes is of importance.

We provided new point of view on the *P. falciparum* confirming main conclusions on the population structure in Cambodia: 1) *P. falciparum* population is fragmented in subpopulations and admixed populations and 2) gene flows are present over the country. The present study covers all areas were transmission of *P. falciparum* is active in Cambodia. The uneven distribution of most alleles suggests that the parasite population is fragmented. Allele frequency, genotype redundancy, *Fst* values and clustering all together confirmed the presence of subpopulations that would have restricted geographic distribution such as in the case in Pailin and both Northern and Southern region of Battambang. These subpopulations might correspond to the KH2, KH3 and KH4 previously described in Cambodia (Miotto et al. 2013). The KH2, KH3 and KH4 subpopulation are carrying preferentially K13 mutant alleles associated to artemisinin resistance (Ariey et al. 2014). We did not observe any association between the K13 mutant alleles and our 23 alleles. This was not surprising considering that C580Y, the most frequent allele, is present in isolates belonging to subpopulation KH2, KH3 and to the admixed population KHA (Ariey et al. 2014)

Fst values were high in Ratanakiri. Accordingly, the level of redundancy was low and we did not find and conserved clusters here, likely due to the high prevalence of the KH1 subpopulation. In fact, the KH1 population was shown to have a high of heterozygosity (Miotto et al. 2013). This subpopulation is composed of individuals carrying the K13 wild type allele. This was confirmed by the geographic distribution of the K13 alleles and the distribution of drug sensitivity in samples from that region (Fig. 4). Allele frequency revealed that new subpopulations could exist in the North and in the South Cambodia. These two regions were not explored by Miotto and collaborators (Miotto et al. 2013). The clustering method showed that isolates from group 3 were restricted to Southern Cambodia (Fig. 7). It might represent a subpopulation localized between health centers of Koh Slar and Takavit. The BC08 reference allele (C) was highly specific in this population. The increase of average *Fst* value from North-Western Cambodia to South-Eastern

Cambodia suggested that diversity increases along that axis, probably due to the distribution of the KHA admixed population described by Miotto et al (Miotto et al. 2013). This admixed population could be represented by group 1, 2 and 4 obtained by clustering as they were distributed all over the country. The center of gravity of these groups was located in the region of Kratie. Interestingly, none of them were detected in Ratanakiri. This group illustrates the ongoing gene flow that supports the dispersal of artemisinin resistance K13 alleles from the West to the East part of Cambodia.

We showed that the barcoding approach based of microsphere and LUMINEX technology is well adapted for epidemiological study but the identification of subpopulations of *P. falciparum* in Cambodian remains challenging.

CONFLICT OF INTEREST

None to be declared.

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Authors contribution

DM, NK and EC design the study. SK, SS, SL, and DM initiated and coordinated sample collection. PC performed *in vitro* susceptibility to antimalarial drugs assays. NK design probes for genotyping. NK, DD, CK, LM, MT performed genotyping. PR and EC develop the algorithm for genotype construction. RF provides critical comments and helpful discussion. NK, AD, CR and EC contribute to data analysis and population study. All authors contribution actively to the manuscript.

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TABLES

Table 1. PCR and LDR conditions. NV for not valid PCR. Eight LUMINEX assys were negative LUMINEX. Assay#7 was rejected because one allele only was not detected (Pos/Neg). LUMINEX detection was performed after microsphere hybridization and ligation reaction. ID of microspheres that were used for several assays are in bold.

	Tm (^{0}C)						Multiplex	
ID	Primary PCR	Nested PCR	LDR	MTAG_ID	LUMINEX	Barcode	PCR	LDR
Assay #1	55	60	58-60	22/25	Neg	NA	NA	NA
Assay #2	55	60	58-60	26/27	Neg	NA	NA	NA
Assay #3	52	59	59	**28**/29	Pos	BC 01	1	1
Assay #4	55	58	59	33/34	Pos	BC 02	2	1
Assay #5	55	58	59	35/36	Pos	Neg	NA	NA
Assay #6	56	60	58-60	38/39	Neg	NA	NA	NA
Assay #7	53	58	58-60	42/43	Pos/Neg	NA	NA	NA
Assay #8	52	58	60	44/45	Pos	BC 03	3	2
Assay #9	52	58	59	**12/13**	Pos	BC 04	3	1
Assay #10	52	58	58-60	14/15	Neg	NA	NA	NA
Assay #11	52	59	58-60	18/19	Neg	NA	NA	NA
Assay #12	52	59	59	20/21	Pos	BC 05	1	1
Assay #13	52	59	59	30/37	Pos	BC 06	1	1
Assay #14	Neg	NA	NA	NA	NA	NA	NA	NA
Assay #15	52	55	59	46/47	Pos	BC 07	4	1
Assay #16	52	58	59	48/51	Pos	BC 08	3	1
Assay #17	Neg	NA	NA	NA	NA	NA	NA	NA
Assay #18	Neg	NA	NA	NA	NA	NA	NA	NA
Assay #19	52	55	59	52/53	Pos	BC 09	4	1
Assay #20	52	58	60	**12/13**	Pos	BC 10	3	2
Assay #21	Neg	NA	NA	NA	NA	NA	NA	NA
Assay #22	52	58	58-60	14/15	Neg	NA	NA	NA
Assay #23	52	58	58-60	26/27	Neg	NA	NA	NA
Assay #24	52	55	60	**28**/78	Pos	BC 11	4	2

Table 2. Major features of the Single Nucleotide Polymorphisms selected for LUMINEX assay. A set 11 SNP has been selected from 24 SNPs validated by Daniels et al (Daniels et al. 2008). NRAF value from three geographic areas and the global MAF were recovered from MalariaGEN version 2 beta-release. Genome position was evaluated according to version 3. Location between core and subtelomere was performed according to PlasmoDB v11.1 and unpublished method. Valid SNPs are highlighted in grey. Frequency of valid alleles was calculated based on Tab. 1 data.

Daniel *et al.*, 2008		MalariaGEN				Study		Mutation				Genome information					
Assay	**SNP ID#**	**WAF**	**WSEA**	**ESEA**	**MAF**	**ID**	**freq**	**Type**	**REF**	**ALT**	**Mutant**	**Chrom.**	**Loc.**	**Genes**	**Gene ID actual**	**Position**	**Gene Name**
1	Pf_01_000130573	0.701	0.438	0.560	0.385	NV	NV	NSY	C	T	A168T	1	core	PFA0145c	PF3D7_0102900	130339	aspartate--tRNA ligase
2	Pf_01_000539044	0.949	0.768	0.816	0.102	NV	NV	SYN	G	A	S173	1	sub	PFA0670c	PF3D7_0113900	537322	Plasmodium exported protein (hyp8)
3	Pf_02_000842803	0.333	0.403	0.543	0.349	BC01	0,56	SYN	C	T	N1004	2	sub	PFB0935w	PF3D7_0220800	842805	cytoadherence linked asexual protein
4	Pf_04_000282592	0.160	0.359	0.398	0.266	BC02	0,36	NSY	T	C	N233S	4	core	PFD0250c	PF3D7_0405100	276127	Sec24 subunit b (SEC24b)
5	Pf_05_000931601	0.305	0.001	0.002	0.157	NV	NV	NSY	C	G	P7739A	5	core	PFE1120w	PF3D7_0522400	931606	conserved Plasmodium protein
6	Pf_06_000145472	0.341	0.421	0.524	0.387	NV	NV	NSY	C	G	S1104T	6	core	PFF0175c	PF3D7_0603600	145475	conserved Plasmodium protein
7	Pf_06_000937750	NA	NA	NA	NA	NV	NV	SYN	A	G	G300	6	core	PFF1105c	PF3D7_0623000	937752	chorismate synthase (CS)
8	Pf_07_000277104	0.102	0.429	0.289	0.250	BC03	0,4	SYN	G	A	L2389	7	core	MAL7P1.19	PF3D7_0704600	221722	ubiquitin transferase, putative
9	Pf_07_000490877	0.363	0.658	0.601	0.333	BC04	0,65	SYN	A	T	A233	7	core	PF07_0040	PF3D7_0709700	435497	lysophospholipase, putative
10	Pf_07_000545046	0.530	0.711	0.571	0.482	NV	NV	SYN	C	T	P435	7	core	PF07_0047	PF3D7_0711000	489666	AAA family ATPase, CDC48
11	Pf_07_000657939	NA	NA	NA	NA	NV	NV	SYN	T	C	D73	7	core	MAL7P1.57	PF3D7_0713000	602559	rifin (RIF)
		NA	NA	NA	NA	NV	NV	SYN	T	C	D76	4	Sub	PFD0134c(p)	PF3D7_0402700	163280	
12	Pf_07_000671839	0.470	0.616	0.365	0.480	BC05	0,3	NSY	G	A	S692N	7	core	MAL7P1.65	PF3D7_0713500	616459	conserved Plasmodium protein,
13	Pf_07_000683772	0.418	0.419	0.404	0.335	BC06	0,21	SYN	C	T	G3129S	7	core	PF07_0053	PF3D7_0713900	628392	conserved Plasmodium protein,
14	Pf_07_000792356	0.264	0.366	0.308	0.274	NV	NV	SYN	A	C	P203P	7	core	PF07_0070	PF3D7_0716900	736978	drug metabolite transporter, putative
15	Pf_07_001415182	NA	NA	NA	NA	BC07	0,4	NSY	C	A	Q584K	7	sub	MAL7P1.176	PF3D7_0731500	1359804	erythrocyte binding antigen-175
		NA	NA	NA	NA		0,41	NSY	C	G	Q584E						
16	Pf_08_000613716	0.233	0.439	0.856	0.459	BC08	0,81	NSY	C	A	F2558L	8	core	PF08_0089	PF3D7_0812100	612596	conserved Plasmodium protein
17	Pf_09_000634010	0.413	0.357	0.300	0.416	NV	NV	NSY	C	T	S61N	9	core	PFI0725c	PF3D7_0914800	634019	GINS complex subunit Psf3, putative
18	Pf_10_000082376	NA	NA	NA	NA	NV	NV	NON	A	T	NON	10	sub	NV	NV	82375	NV
19	Pf_10_001403751	NA	NA	NA	NA	BC09	0,6	NSY	A	C	T1106P	10	core	PF10_0344	PF3D7_1035300	1402510	glutamate-rich protein (GLURP)
20	Pf_11_000117114	0.158	0.589	0.531	0.384	BC10	0,43	NSY	G	A	S597F	11	sub	PF11_0037	PF3D7_1102500	119497	Plasmodium exported protein (PHISTb)
21	Pf_11_000406215	0.473	0.729	0.661	0.354	NV	NV	NSY	A	C	N1036H	11	core	PF11_0108	PF3D7_1110200	408600	pre-mRNA-processing factor 6
22	Pf_13_000158614	NA	NA	NA	NA	NV	NV	SYN	T	C	T35	13	core	MAL13P1.15	PF3D7_1303000	158412	conserved Plasmodium protein
23	Pf_13_001429265	0.000	0.995	0.999	0.396	NV	NV	NSY	T	G	N3191T	13	core	MAL13P1.176	PF3D7_1335300	1429067	reticulocyte binding protein 2+
24	Pf_14_000755729	0.000	0.820	0.958	0.426	BC11	0,98	NSY	G	T	R736I	14	core	PF14_0177	PF3D7_1417800	755731	DNA replication factor MCM2

Table 3. Decision rules for genotyping. Primer 1 and Primer 2 correspond to the two measurements perfromed using microspheres for one locus. The signal for one primer could Positive, Negative or uncertain. Manual curration was possible at this step. The decision laws are applyied afterwards. The decision Other means that the allele at that position is none of the two tested with microspheres. This result should be confirmed by two more measurments. This was true for about 40 % of the samples at BC07 locus. Not valid decision means that the experiment should performed again or the sample should be rejected from the analysis.

Sample i	Primer 1	Primer 2	Decision
A	Positive	Positive	Mixture
B	Positive	Negative	Primer 1
C	Negative	Positive	Primer 2
D	Negative	Negative	Other
E	Uncertain	Positive	Primer 2
F	Uncertain	Negative	Not Valid
G	Positive	Uncertain	Primer 1
H	Negative	Uncertain	Not Valid
I	Uncertain	Uncertain	Not Valid

Figure legends

Figure 1. The PCR-LDR-FMA (Polymerase Chain Reaction-Ligase Detection Reaction Fluorescent Microsphere-based Assay) experimental procedure. We amplify a locus by PCR to test the presence of a specific allele (top of schema: blue or red). The allele-specific primer is composed of two parts. Part2 hybridize on the DNA (green). Part 1 is recognized by the microsphere (yellow or black). The locus-specific is coupled to biotin at 3-prime which will be recognized by streptavidine-R phycoreythrine dye.

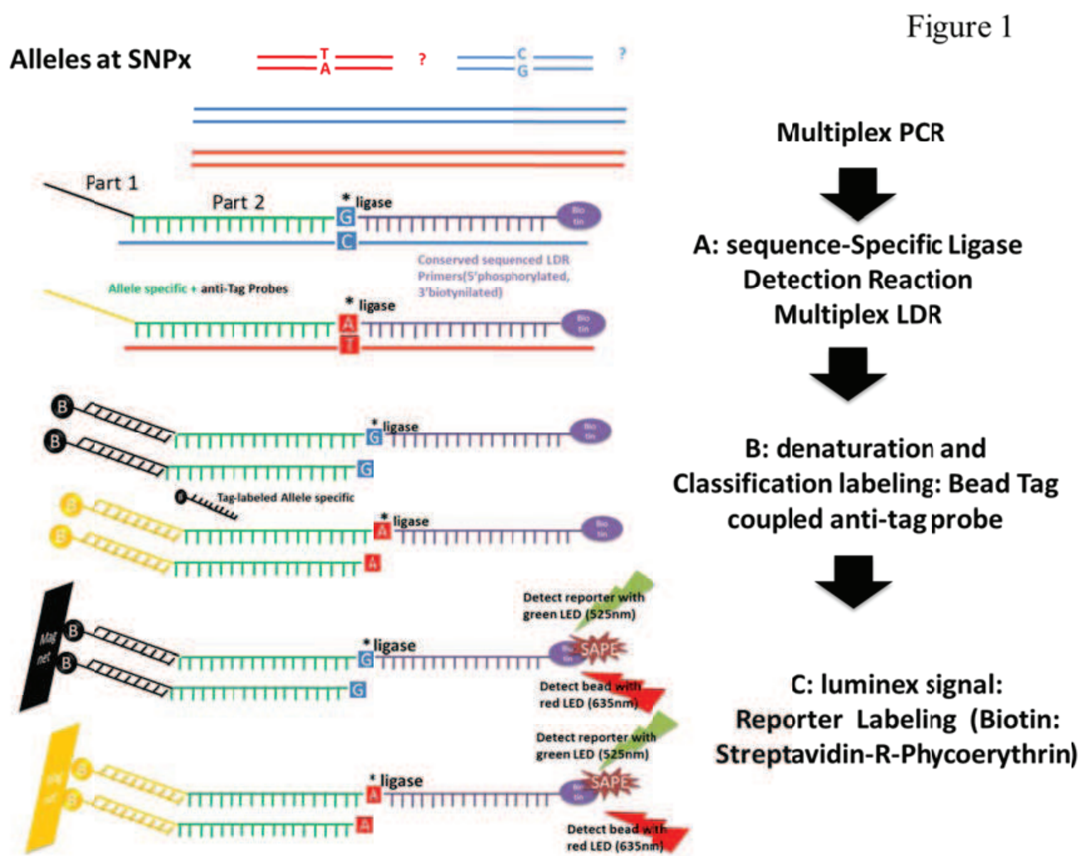


Figure 2. Distribution of Streptavidin, R-Phycoerythrin Conjugate (SAPE) fluorescence intensity. Distribution were drawn from a large range of values, between 500 and 800 depending on the allele. a) Standard distribution observed for BC01 reference allele assessment. The distribution is bimodal. The population on the left correspond to negative samples. The population on the right correspond to positive samples. b) Distribution presenting extreme values (Altered allele for BC01 locus). Two small populations appears for null values and for very high fluorescent values. These samples were generally characterized as uncertain in our clustering approach. c) Distribution presenting a low range of variation (reference allele for BC09 locus). In some cases, the assay provide low fluorescence values for the positive samples. d) Distribution presenting high values for positive samples (altered allele for BC09 locus). e) Schematic representation of the analysis performed automatically by our detection algorithm. We assume that the signal of a reading can be distributed among two populations corresponding either to the negative samples or to the positive one. The distribution of the two populations was expected to have a Normal distribution.

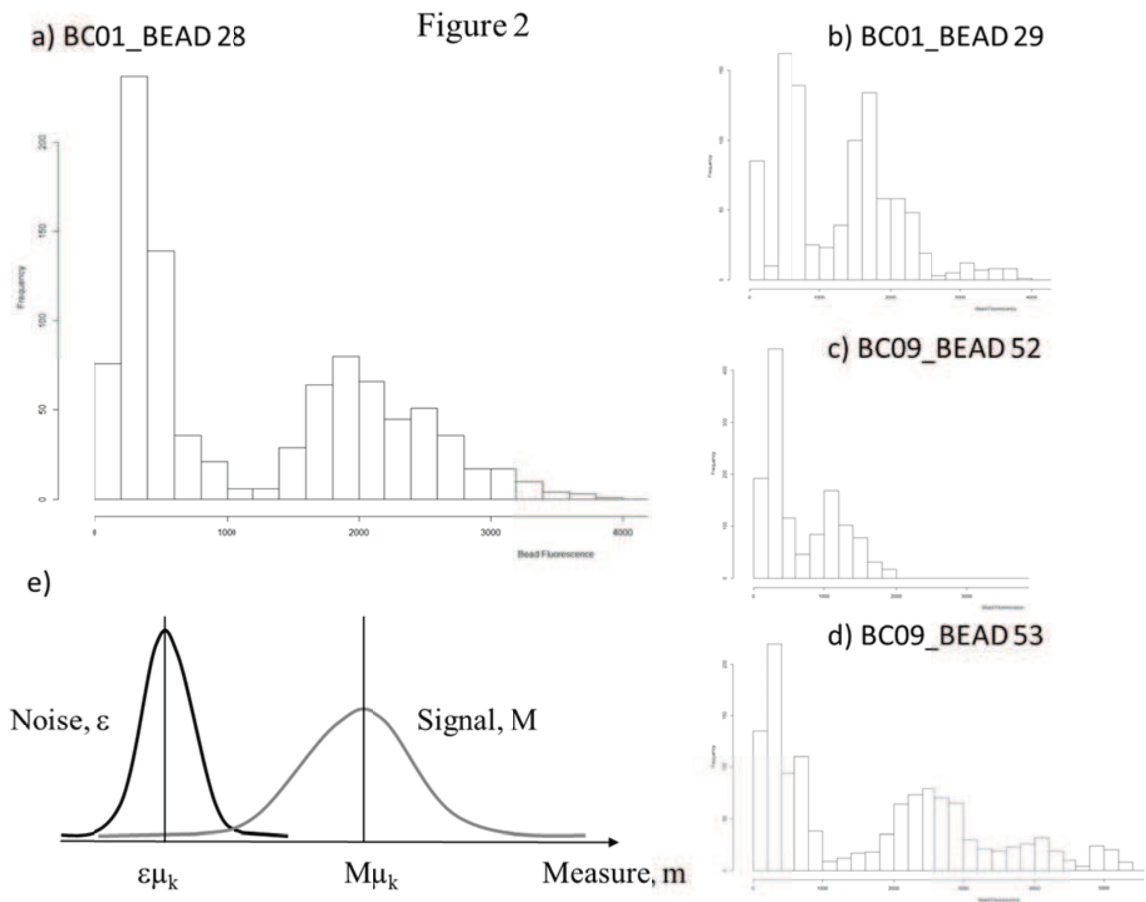


Figure 3. Allele distribution among 15 health centers (HC: Health centre, RH: Reference Hospital) in Cambodia. 1: Tasanh, 2: Pailin RH, 3: Ou Chra HC, 4 : Sampoev Loun RH, 5 : Battambang RH, 6 : Anlong Veng RH, 7 : Trapaing Prasat HC, 8: Veun Say HC, 9: Keov Seima HC, 10: Khsim HC, 11: Chhouk HC, 12: Koh Sla HC, 13: Takvit HC, 14: Oral HC, 15: Promoy HC

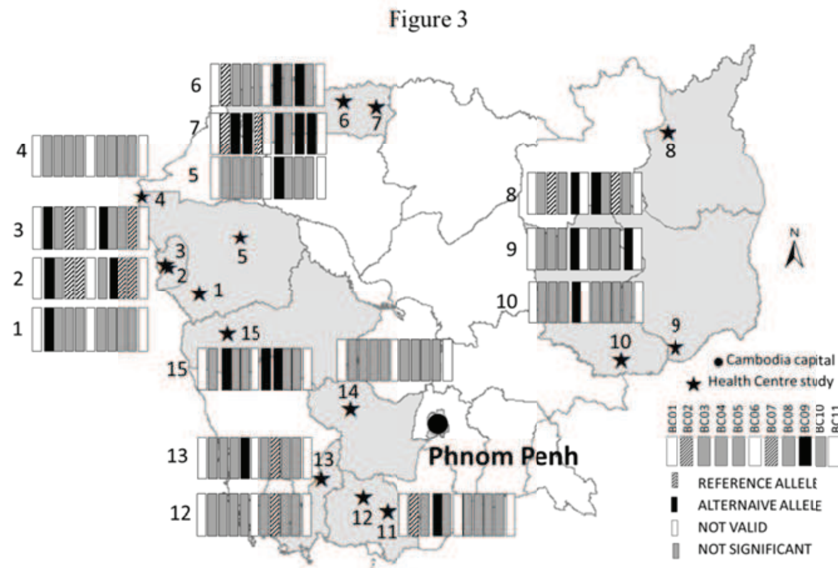


Figure 4. Distribution of drug sensitivity among samples over Cambodia. Sampling areas were groups at each cardinal points. East, North, South, West. Boxplot represent median and quartile distribution. *P-values* were obtained from median and standard deviation comparison.

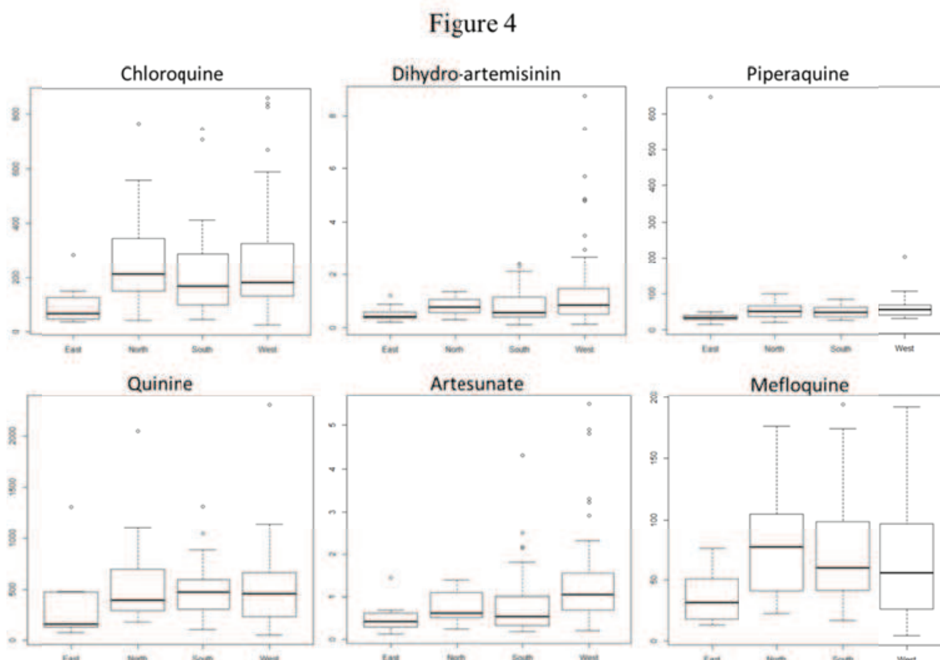


Figure 5. Correspondence analysis for the 23 alleles tested for the 11 loci. Each reference (REF) and altered (ALT) alleles are represented.

Figure 5

Figure 6. Intra genetic diversity (average *Fst*) among the Samples from 15 health centers (HC: Health centre, RH: Reference Hospital). 1: Tasanh, 2: Pailin RH, 3: Ou Chra HC, 4 : Sampoev Loun RH, 5 : Battambang RH, 6 : Anlong Veng RH, 7 : Trapaing Prasat HC, 8: Veun Say HC, 9: Keov Seima HC, 10: Khsim HC, 11: Chhouk HC, 12: Koh Sla HC, 13: Takvit HC, 14: Oral HC, 15: Promoy HC. The average *Fst* value was calculated among all individuals isolated from a specific health center.

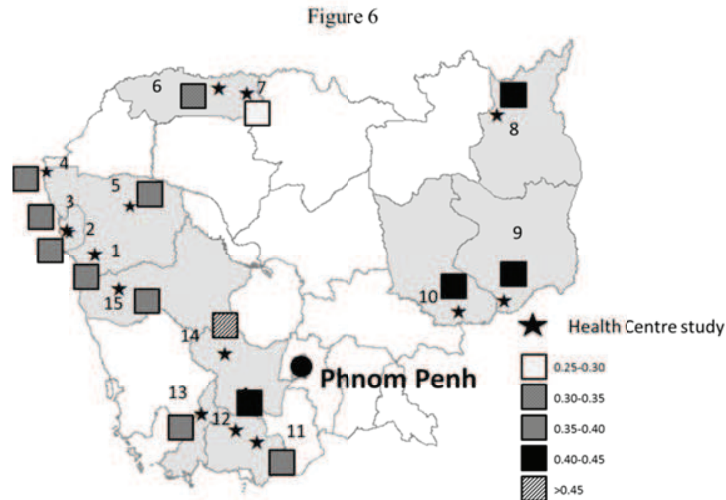
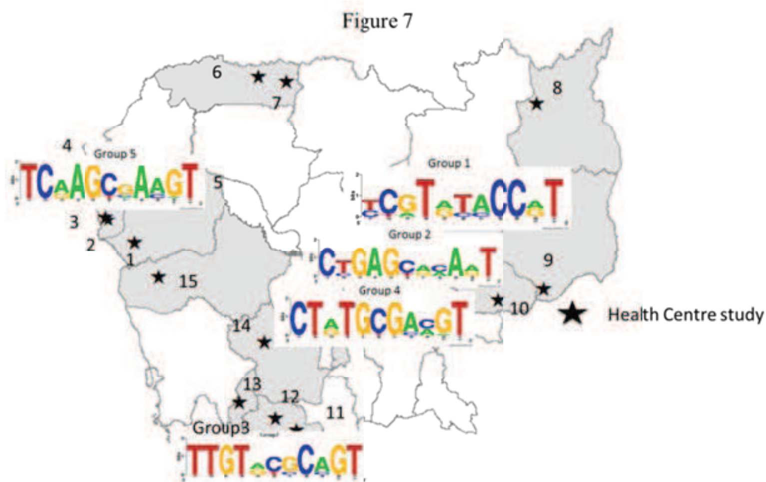


Figure 7. Distribution of stable clusters in 15 health centres (HC: Health Centre, RH: Referece Hospital)over Cambodia . 1: Tasanh, 2: Pailin RH, 3: Ou Chra HC, 4 : Sampoev Loun RH, 5 : Battambang RH, 6 : Anlong Veng RH, 7 : Trapaing Prasat HC, 8: Veun Say HC, 9: Keov Seima HC, 10: Khsim HC, 11: Chhouk HC, 12: Koh Sla HC, 13: Takvit HC, 14: Oral HC, 15: Promoy HC. Small Group of samples cluster together whatever clustering approaches. Effective per group:



SUPPLEMENTARY MATERIALS

Table S1. Geographic distribution of samples and allele frequency. We collect blood samples from *P. falciparum* positive patients from 15 health centers or hospitals covering all areas where parasite transmission is active. We provide frequency of the total number of isolates (418) and of valid SNPs (221) per health centers. The frequency of each allele is given for the 11 loci which were positive for LUMINEX detection. The presence of Kelch-propeler domain altered allele was assessed by PCR and sequencing. The frequencies of wild type individuals and of the two major alleles C580Y and R539T are given. The other alleles (Oth) present at low frequency were N458Y, Y493H, I543T, P553L and V568G. They were pooled together. We measure routinely the drug sensitivity for patients presenting high parasitemia (>2%). The number of CI50 measurements given for chloroquine (CQ), piperaquine (PIP), Quinine (QN), Artesunate (ART), Mefloquine (MF) and Dihydro-artemisinin (DHA).

						BC01		BC02		BC03		BC04		BC05		BC06		BC07			BC08		BC09		BC10		BC11		Kelch allele					CI50						
ID	Regions	Province	Health center HC or hospital RH	Total	Valid	REF C	ALT T	REF T	ALT C	REF G	ALT A	REF A	ALT T	REF G	ALT A	REF C	ALT T	REF C	ALT A	ALT G	REF C	ALT A	REF A	ALT C	REF G	ALT A	REF G	ALT T	Exp.	WT	C>Y	R>T	Other	CQ	PIP	QN	ART	MF	DHA	
1	North	Oddar Meanchey	Anlong Veng RH	18	10	3	7	10	0	8	2	3	7	9	1	9	1	4	4	2	1	9	1	9	6	4	0	10	3	1	2	0	0	1	1	1	1	1	1	0.10
2	West	Battambang	Battambang RH	21	8	2	6	4	4	4	4	2	6	7	1	6	2	1	1	6	2	6	1	7	5	3	0	8	2	2	0	0	0	2	3	3	3	3	3	0.38
3	South	Kampot	Chhouk HC	26	17	8	9	16	1	13	4	2	15	12	5	14	3	2	6	9	4	13	7	10	9	8	1	16	6	0	4	1	0	8	9	9	8	9	8	0.47
4	East	Mondulkiri	Kaev Seima HC	9	5	2	3	3	2	4	1	2	3	1	4	4	1	2	2	1	1	4	1	4	0	5	0	5	2	1	0	0	1	1	1	1	1	1	1	0.20
5	East	Kratie	Khsim HC	17	12	8	4	9	3	6	6	2	10	5	7	9	3	4	3	5	3	9	5	7	7	5	0	12	7	3	3	0	1	4	5	4	4	5	5	0.42
6	South	Kampot	Koh Slar HC	23	15	7	8	12	3	9	6	3	12	9	6	11	4	1	9	5	10	5	6	9	8	7	0	15	5	1	3	1	0	7	7	7	7	7	7	0.47
7	South	Kampong Speu	Oral HC	24	3	0	3	1	2	2	1	2	1	2	1	2	1	0	2	1	1	2	1	2	1	2	0	3	0	0	0	0	0	2	1	1	2	1	2	0.67
8	West	Pailin	Ou Chra HC	20	17	5	12	5	12	7	10	12	5	14	3	14	3	4	2	11	1	16	8	9	15	2	0	17	10	3	6	0	1	9	9	9	11	10	11	0.65
9	West	Pailin	Pailin RH	39	27	8	19	11	16	13	14	14	13	23	4	23	4	6	7	14	1	26	20	7	24	3	0	27	25	1	22	2	0	16	13	15	16	16	16	0.59
10	West	Pursat	Promoy HC	56	30	10	20	16	14	13	17	15	15	25	5	24	6	2	16	12	2	28	9	21	17	13	1	29	6	2	4	0	0	16	11	15	16	16	16	0.53
11	West	Battambang	Sampovloun RH	9	5	3	2	4	1	4	1	2	3	4	1	5	0	1	3	1	0	5	1	4	2	3	0	5	3	0	2	1	0	2	2	2	2	2	2	0.40
12	South	Kampong Som	Takavit HC	30	19	11	8	13	6	15	4	4	15	4	15	17	2	1	7	11	8	11	7	12	10	9	0	19	5	1	4	0	0	15	15	15	15	14	15	0.79
13	West	Battambang	Tasanh HC	30	5	1	4	0	5	3	2	3	2	5	0	4	1	1	1	3	0	5	1	4	4	1	0	5	3	1	1	0	1	1	1	1	1	1	1	0.20
14	North	Oddar Meanchey	Trapaing Prasat HC	44	28	19	9	26	2	12	16	2	26	25	3	23	5	5	18	5	3	25	6	22	9	19	0	28	12	3	5	4	0	20	21	20	21	21	21	0.75
15	East	Rattanakiri	Veurn Say HC	52	20	11	9	12	8	20	0	10	10	10	10	10	10	7	7	6	4	16	14	6	8	12	3	17	12	12	0	0	0	5	5	5	5	5	5	0.25

Table S2. Primers sequences for PCR reactions corresponding to 20 of the 24 SNPs selected for barcode detection.

ID	Barcode	Orientation	Primary PCR	Length (bp)	Nested PCR	Length (bp)
Assay#1	NA	Forward Reverse	AAAATGAAGCAGCAAAAG ATCTAATATACATTGAAGGGAATA	350	GATTGTGAAAATGAGAAGAGAGAAA AAAGCAATCGAACCCTTTG	149
Assay#2	NA	Forward Reverse	AAAATTCACAATGATTTAACAC TTTTTCTGAGTAAAGATCAGC	285	AAAACAAATGGCATCCACTG TGATAAAACCAAGAAGTCCAAGA	164
Assay#3	BC01	Forward Reverse	TGGAAATACACAATTCAATG CGAATGTTTTTCCATATTTT	273	TTCCAAAACTATGTTTGCTGCT TGCAGTGGTACTTGTTGCTACC	124
Assay#4	BC02	Forward Reverse	CCAACCAACGAACACAAATAC TGGTTGACTGTTATTGGGGTA	250	AGGAAAATGCTCCGGTAACT GGTTCATATTATTTGGTGACTCG	120
Assay#5	NA	Forward Reverse	TCAGGAATTTGAAGAACATT TTTCTTTCCTCATTTCTGTT	215	TGAAAATAATGATGAGAATGATGTC ATGTGGGGAACTATTAATGGAG	148
Assay#6	NA	Forward Reverse	TGACAAGAAAACAAAGAATG ATCAACAAAAGCTGATGAAT	298	TGTGCAAGAAAAGTAGGAAATG TGTGTCCCCTCTTTTTCTGT	148
Assay#7	NA	Forward Reverse	TGAATGTAATATAAATCAGGTTG GGCTGGAATAGATAAAATCA	292	CTGAAAAATCGGATGAATGG GGCTAGCTCAGCTTCCAAT	111
Assay#8	BC03	Forward Reverse	CGAATTTAAGTACCTTAGGAAA TCATAAAGTTTTTATTGTCTTCA	297	TCACAACGTCCATATGTTGAA TCATTATCACCTACTTTCTGTACCA	176
Assay#9	BC04	Forward Reverse	GAGGATGTATACCATTAGCTG ATCATTCATATGTGGAAACA	164	GATGAGTTAGCAACGAAACCA AACGTAAACCAGGAGTAAGACG	103
Assay#10	NA	Forward Reverse	GTTGTTATATCTTTTGCTATGAA CGTAATGGTTTATAACTATTCTTT	200	AAATCCATCCTTTTATGAATGC TGGATGTATGACAGCTTTTTCTAA	113
Assay#11	NA	Forward Reverse	ATACACTAAACGCAAAACCT TGTTAATTCCTTTTCGATTT	242	CATTATGCGAATGCGATCTA CGTTTATATTGCAACATTTCTTCA	130
Assay#12	BC05	Forward Reverse	CAATGAATATGAATTGGATTT GAACATTAAGTAAAATGTGATCC	287	GATGAAAATGGAACCATTCATAG TGTTGGTCTTTTTCCTCTTCTAA	158
Assay#13	BC06	Forward Reverse	TGACAAACAAGTATATAATAATAAGAG TGTTTTAAAAGTCGTGGATA	385	TGTTGTTGGTGAATACAATGAAA TCGTACCACCATTAACATTTTG	161
Assay#14	NA	NA	NA	NA	NA	NA
Assay#15	BC07	Forward Reverse	CATAAATAAAACTTTCGCTGA ATTTTCAATATCATCTTCTTTACA	241	TGGAATGATTTGAGCAATAGAA AATACCCATGATATCACATTCCA	149
Assay#16	BC08	Forward Reverse	ATCATCTGTATTTTGTTATTATGA GTTAGACAATTTTGCTACACTT	247	AATCTTTTCCAGTTATTTTCTATCCA CATGGGGGTATGTAATTTGG	168
Assay#17	NA	NA	NA	NA	NA	NA
Assay#18	NA	NA	NA	NA	NA	NA
Assay#19	BC09	Forward Reverse	TCACAAACAAATAACAATGAA ACATGTTTTGGACCATCTAC	248	AAAAGCAATTCCACAAGAACC CTGGTGTTTCCTTTTTATTTGG	149
Assay#20	BC10	Forward Reverse	ATCGCATGTAGATTAATATGG AAGGAGCTTCTGTAATACTATTTT	241	AAGAAAATTAAAGAGCATGAATCG CATAAGAACTGCTTCCATCTCC	136
Assay#21	NA	NA	NA	NA	NA	NA
Assay#22	NA	Forward Reverse	AATATATCTGTATTTGCTAACATGA TGTAACAAGGAATGACAAAA	288	TGTGTTTTATTTTTAGTGTGAGCTTT AGAGGATATCCAATAGGGTGCT	119
Assay#23	NA	Forward Reverse	TGTAACAGAAGAAGATTACACG TATCAGCATAATTCGATAAGTG	246	CAATAATGAAAGGTATTCAAACCA TACTTCTTCACACATATCAAAATCG	158
Assay#24	BC11	Forward Reverse	CGATTTAATTACTGTTTTGAGA TTGGTTTACAATTAGTTCTAGC	233	AACAAATCATCAATTAAGTCATCC TGAGGAATAGGTTCATATGCTG	114

Table S3. Primer sequences for LDR (Ligation Detection reaction) performed

ID	Barcode	Allele	Microsphere ID	LDR PRIMER		Conserved Primer
				Part 1	Part 2	
Assay#1	NA	REF	MTAG-A022	CAAACAAACATTCAAATATCAATC	GATAAAAATGTAGGTGATGTAAAAGATG	[Phos]CATATAATGAAGAAAATTTATTAGGAAAGA[BtnTg]
		ALT	MTAG-A025	CTTTCTTAATACATTACAACATAC	GATAAAAATGTAGGTGATGTAAAAGAT**A**	
Assay#2	NA	REF	**MTAG-A026**	TACATTCAACACTCTTAAATCAAA	GCTTTCCTATCAACCGTTTCC	[Phos]TTAGCCTTCACAGTTGTAATGTTATCT[BtnTg]
		ALT	**MTAG-A027**	TAACTTACACTTAACTATCATCTT	GCTTTCCTATCAACCGTTTC**T**	
Assay#3	BC01	REF	**MTAG-A028**	CACTTAATTCATTCTAAATCTATC	TTTCAAATGTTATTTTCAACTATGTTAAGTAAC	[Phos]GATGCAAATAATCTTGATAAAGTATATGG[BtnTg]
		ALT	MTAG-A029	TACTACTTCTATAACTCACTTAAA	TTTCAAATGTTATTTTCAACTATGTTAAGTAA**T**	
Assay#4	BC02	REF	MTAG-A033	ACTACTTATTCTCAAACTCTAATA	GAAAAAAATAATTTGAACAATAAAACTTATAATAA	[Phos]CATGAACGAGTCACCAAATAATATG[BtnTg]
		ALT	MTAG-A034	ACTTATTTCTTCACTACTATATCA	GAAAAAAATAATTTGAACAATAAAACTTATAATA**G**	
Assay#5	NA	REF	MTAG-A035	CATCTTCATATCAATTCTCTTATT	TGATGTTCATATTCACAATGATCAAC	[Phos]CACCCGAACTAAATCGCTCC[BtnTg]
		ALT	MTAG-A036	ATTAAACAACTCTTAACTACACAA	TGATGTTCATATTCACAATGATCAA**G**	
Assay#6	NA	REF	MTAG-A038	ATTCAATACTATCTAACACTTACT	GGATGTTATTAAAAATGAAGAGAAGCATAG	[Phos]TAAGAAACATTTTAATATTTTACAGAAAAAGAGG[BtnTg]
		ALT	MTAG-A039	ACAAATATCTAACTACTATCACAA	GGATGTTATTAAAAATGAAGAGAAGCATA**C**	
Assay#7	NA	REF	MTAG-A042	CACTACACATTTATCATAACAAAT	AAGGAGATAGTGTTGGGGGC	[Phos]ATTGCTACATGCATTATACAAAATCC[BtnTg]
		ALT	MTAG-A043	AACTTTCTCTCTCTATTCTTATTT	AAGGAGATAGTGTTGGGGG**T**	
Assay#8	BC03	REF	MTAG-A044	TCATCACTTTCTTTACTTTACATT	TGATGAAAGCCACCGAACTC	[Phos]ATATTTATGGATGAACATTATATTAATAAAGATAT[BtnTg]
		ALT	MTAG-A045	TACACAATATTCATCATAACTAAC	TGATGAAAGCCACCGAACT**T**	
Assay#9	BC04	REF	**MTAG-A012**	CATAATCAATTTCAACTTTCTACT	CCATCATATAAATATTTCTATATTCCATTAGCT	[Phos]AAATTCTTAGGAAGCTTTTTTCCAAG[BtnTg]
		ALT	**MTAG-A013**	CAAATACATAATCTTACATTCACT	CCATCATATAAATATTTCTATATTCCATTAGC**A**	
Assay#10	NA	REF	**MTAG-A014**	AATTTCTTCTCTTTCTTTCACAAT	TACGTAATGTTGTCTTAAGCCCG	[Phos]TTTAGTGATACCATACCTAATTTAAGTAGAGAAG[BtnTg]
		ALT	**MTAG-A015**	TACTTCTTTACTACAATTTACAAC	TACGTAATGTTGTCTTAAGCCCA	
Assay#11	NA	REF	MTAG-A018	ACACTTATCTTTCAATTCAATTAC	AAATGAAAGAAGTGATGCAACAATTTGAT	[Phos]GATCGTGCCGCACAACGA[BtnTg]
		ALT	MTAG-A019	ATACTTTACAAACAAATAACACAC	AAATGAAAGAAGTGATGCAACAATTTGA**C**	
Assay#12	BC05	REF	MTAG-A020	CTTTCTCATACTTTCAACTAATTT	AATGGAAAATTTTGATGATATTTTATTAAG	[Phos]TGAAAATGAAAAAGAATTATCTTCATATAAT[BtnTg]
		ALT	MTAG-A021	TCAAACTCTCAATTCTTACTTAAT	AATGGAAAATTTTGATGATATTTTATTAA**A**	
Assay#13	BC06	REF	MTAG-A030	CTTAACATTTAACTTCTATAACAC	AAATAACAATGAACATCATCATGATG	[Phos]GTTCAGTTATTCCAAATAATTTTTGTAATAA[BtnTg]
		ALT	MTAG-A037	TACAACATCTCATTAACATATACA	AAATAACAATGAACATCATCATGAT**A**	
Assay#14	NA	REF	NA	NA	NA	NA
		ALT				
Assay#15	BC07	REF	MTAG-A046	TTAAACAATCTACTATTCAATCAC	AAATTCAAATTATGTTCACAGGAATAAAC	[Phos]AAAATGATAAGCTTTTTCGTGATGA[BtnTg]
		ALT	MTAG-A047	TCTCTTTAAACACATTCAACAATA	AAATTCAAATTATGTTCACAGGAATAAA**A**	
Assay#16	BC08	REF	MTAG-A048	AATCAACACACAATAACATTCATA	ACCTTCCATATCTAAAAAAACTTCATTC	[Phos]AAAATCATAGACAAAAAAAAAACAGTTTC[BtnTg]
		ALT	MTAG-A051	CAATTTACATTTCACTTTCTTATC	ACCTTCCATATCTAAAAAAACTTCATT**A**	
Assay#17	NA	REF	NA	NA	NA	NA
		ALT				
Assay#18	NA	REF	NA	NA	NA	NA
		ALT				
Assay#19	BC09	REF	MTAG-A052	TTCTTCATTAACTTCTAATCTTAC	CCTACATTAAATGAAAATGAAAACGTTA	[Phos]CTCCCAAACCATCTGAAGGT[BtnTg]
		ALT	MTAG-A053	TTAACAACTTATACAAACACAAAC	CCTACATTAAATGAAAATGAAAACGTT**C**	
Assay#20	BC10	REF	**MTAG-A012**	CATAATCAATTTCAACTTTCTACT	CAAAATATCAACAAGAAAAACATAATTACTC	[Phos] TTGGATGAAATTTCTTGATGAATATAA [BtnTg]
		ALT	**MTAG-A013**	CAAATACATAATCTTACATTCACT	CAAAATATCAACAAGAAAAACATAATTACT**T**	
Assay#21	NA	REF	NA	NA	NA	NA
		ALT				
Assay#22	NA	REF	**MTAG-A014**	AATTTCTTCTCTTTCTTTCACAAT	GTAAATATTTTAGTGAAGATTATTTTTGGACT	[Phos]CAAGCTAATATAGGTCCATTGTGTATT[BtnTg]
		ALT	**MTAG-A015**	TACTTCTTTACTACAATTTACAAC	GTAAATATTTTAGTGAAGATTATTTTTGGACC	
Assay#23	NA	REF	**MTAG-A026**	TACATTCAACACTCTTAAATCAAA	AACAAAATGGATGATTTCGTATATACA	[Phos]TGCTGGAGGAGTTGTTTGTT[BtnTg]
		ALT	**MTAG-A027**	TAACTTACACTTAACTATCATCTT	AACAAAATGGATGATTTCGTATATAC**C**	
Assay#24	BC11	REF	**MTAG-A028**	CACTTAATTCATTCTAAATCTATC	AATTAGAAAATACACAAAATTATCAAAAAAG	[Phos] AATTGAAAATTTAAAAAATGTTATTGTTTC [BtnTg]
		ALT	MTAG-A078	TTTACAAATCTAATCACACTATAC	AATTAGAAAATACACAAAATTATCAAAAAA**T**	

DISCUSSION GENERALE

L'épidémiologie moléculaire est une démarche indispensable pour comprendre la dynamique des populations parasitaires et informé les programmes d'élimination du paludisme. Cette méthodologie repose essentiellement sur l'identification de marqueurs spécifiques et sur un échantillonnage représentatif. Le développement des techniques moléculaires permet de disposer d'un large panel de méthodes utiles pour étudier les variations du génome pouvant être en relation avec la pathogénicité du parasite. Dans le cadre de ma thèse, je me suis concentrée sur plusieurs objectifs ayant trait à l'émergence et à la diffusion de caractères de résistance aux antipaludéens, principal problème rencontré au Cambodge. En effet, le Cambodge est reconnu comme l'épicentre de l'émergence des souches multi résistantes.

Etude des marqueurs des résistances.

Au cours de ma thèse j'ai pu étudier le polymorphisme de deux marqueurs impliqués dans la résistance aux antipaludiques : au niveau du gène *dhfr* codant pour la dihydrofolate reductase et au niveau du gène *mdr* codant pour un transporteur ABC associé à la famille des mutlti-drug resistance. Le gène *dhfr* a été étudié chez *P. malariae* et *P. ovale*. Il est impliqué dans la résistance à la pyrimethamine. Le gène *mdr* a été étudié chez *P. vivax*. Il est impliqué dans la résistance à la méfloquine. L'approche moléculaire pour étudier les mécanismes de résistance mis en place par ces 3 espèces était une des alternatives possibles en l'absence de système permettant de cultiver ces parasites (ce qui n'est pas le cas pour *P. falciparum).*
Nous avons considéré dans ce travail que les gènes orthologues décrits chez *P. vivax*, *P. malariae* et *P. ovale*, remplissent les mêmes fonctions que celles décrites chez *P. falciparum*, car les génomes des *Plasmodium* infestant l'homme sont très conservés. L'ordre des gènes (synténie) est conservé. Il y a quelques insertion/délétions mais elles concernent essentiellement les familles multigéniques spécifiques à chaque espèce. Ces informations sont accessibles à travers une base de données comme PlasmoDB. Les séquences des gènes peuvent par contre varier de façon importante entre les espèces. Le premier enjeu de l'épidémiologie moléculaire est donc de caractériser ces gènes pour chaque espèce. Ce travail s'est fortement appuyé sur le séquençage systématique de nombreuse espèce, même si les génomes de *P. malariae* et *P. ovale* ne sont pas encore publiés. On s'aperçoit que les séquences des protéines sont modifiées. Dans ces conditions, il n'est pas évident que les mutations trouvées dans les gènes soient réellement impliquées dans la résistance. A travers l'échantillonnage, l'épidémiologie moléculaire cherche à établir des relations entre certaines mutations et la sensibilité aux drogues.

La même démarche a été appliquée au Cambodge pour l'identification de marqueurs impliqués dans la résistance à l'artémisinine. Des masses de données importantes ont été analysées dès les premiers rapports concernant l'émergence de résistance à l'artémisinine (Cheeseman et al. 2012, Dondorp et al. 2009). Ces études ont été confrontées à un double problème. Le premier problème était dû à la faiblesse de la caractérisation du phénotype de résistance. Celui était fondé sur la clinique (délai de clairance parasitaire au cours des 3 jours de traitement à l'artémisinine seule). Plusieurs études nous ont mis sur la voie qui nous a permis de développer le test RSA (Mok et al. 2011, Saralamba et al. 2011). Ce test a été développé en

adaptant les souches de terrain à la culture *in vitro,* étape délicate qui ne réussit pas toujours. C'est la raison pour laquelle nous avons décidé de poursuivre notre étude vers la caractérisation d'un marqueur moléculaire spécifique de la résistance à l'artémisinine. Cette étude a été rendue possible grâce au développement des techniques de séquençage. Une approche en laboratoire a permis de montrer que l'acquisition de la résistance pouvait être liée à des mutations dans la partie du gène PF3D7_1343700 codant pour le domaine propeller d'une protéine Kelch-like. Le séquençage ensuite de plus de 50 isolats au niveau de la plate-forme de génomique de la Génopôle à l'Institut Pasteur (Paris) nous a permis de corréler le niveau de résistance à l'artémisinine exprimé par le test RSA avec la présence d'allèles spécifique. Il a récemment été montré chez la souche 3D7 que l'introduction de la mutation C580Y était suffisante pour induire la résistance à l'artémisinine (Ghorbal et al. 2014). L'expérience consistant à retirer l'allèle muté d'une souche résistante pour la rendre sensible sera la preuve définitive que le marqueur que nous avons pu caractériser est un marqueur essentiel dans l'acquisition et la diffusion de la résistance à l'artémisinine. Il apparait dans la base de données MalariaGEN version 2 bêta que les allèles caractérisés restent pour l'instant restreints à l'Asie du Sud-Est.

Méthodologie de l'étude

A travers cette thèse, nous avons constaté que l'échantillonnage joue le rôle très important dans l'épidémiologie du paludisme. Dans le premier chapitre, nous avons pu comparer les séquences du gène *dhfr* dans deux espèces différentes *Plasmodium malariae* et *Plasmodium ovale*. Les échantillons provenaient du Cambodge, de Madagascar, de l'Afrique. Dans ces différentes parties du monde, l'utilisation de l'association sulfadoxine-pyriméthamine était différente. Il en est de même pour l'usage de méfloquine dans le cas de l'étude du gène *mdr-1* chez *Plasmodium falciparum* et *Plasmodium vivax*. Les échantillons africains ont été sélectionnés dans la biobanque du Centre National de Référence du Paludisme (CNRP). Il s'agit d'ADN voyageurs. Les échantillons de Madagascar et du Cambodge ont été obtenus à partir cas autochtone. De plus, grâce aux bases de données, nous avons pu récupérer les données disponibles déjà déposés par d'autres groupes de recherche.

Dans le cadre de notre étude sur l'artémisinine, nous avons analysé 941 ADN stockés dans notre biobanque depuis 2001. Nous avons pu comparer l'évolution des allèles du domaine propeller du gène K13 depuis 2001. La résistance à l'artémisinine a été documentée en 2008. Grâce à la biobanque nous avons pu faire une étude rétrospective sur plus de 10 ans. Il est important à noter qu'en 2001, l'Organisation Mondiale de la Santé portait beaucoup d'espoir de l'efficacité des dérivés de l'artémisinine. Ces antipaludiques ont joué un rôle très important dans la vie des populations menacée par le paludisme. L'impact a été spectaculaire chez les enfants moins de 5 ans en Afrique. Dans les travaux publiés, nous avons montré que la résistance à l'artémisinine est exprimée par des isolats présents dès la création de notre biobanque, en 2001. La souche la plus ancienne que nous ayons et qui soient originaire du Cambodge est la souche K1992. Nous avons montré que cette souche qui vient de la région de Pailin est sensible à l'artémisinine.

Le développement d'une approche de typage des souches par barcode doit nous permettre de mieux suivre l'évolution des flux de gènes au sein de la population parasitaire au Cambodge. La question est ancienne. Nous avons dû mettre en place une approche méthodologique fiable qui devait passer par l'utilisation de méthode rapide et par un traitement des données efficace. Nous sommes partis d'un jeu de SNPs déjà validé pour ce type d'approche. Miotto et al (2013) ont depuis montré que la structuration des populations parasitaires chez *Plasmodium falciparum* à l'Ouest du Cambodge était particulière. Plusieurs sous populations parasitaires distinctes ont été observés dans cette région : KH2, KH3 et KH4. Ces populations sont très fortement liées à la résistance à l'artémisinine. Ariey et al (2014) suggèrent qu'une liaison génétique forte existe entre les allèles mutés dans le domaine propeller du gène Kelch13 et ces sous-populations. L'étude que nous avons menée avec 11 SNPs ne nous a pas permis d'établir de relation avec ces sous-populations. Nous avons bien observé une fragmentation de la population parasite mais aucune liaison génétique n'a pu être établie entre les allèles choisis et les allèles Kelch 13. En revanche, la force de l'étude que nous avons menée vient de sa couverture géographique. Les échantillons proviennent de 15 centres de santé répartis dans toutes les zones géographiques où la transmission du parasite est active. Nous avons pu mettre en évidence des flux de gènes sur la base de certains allèles. Ces allèles indiquent que des sous-populations existent aussi au nord et au sud du pays, régions non étudiées jusqu'à présent. Cependant, nous ne disposons pas de marqueurs spécifiques de ces sous-populations. L'étude par barcode sur les isolats depuis 2001 nous permettra de remonté peut être à l'origine de ces sous-population et à l'effet fondateur de l'introduction systématique des dérivés de l'artémisinine. Ainsi, de nombreux défis apparaissent pour déterminer les sous populations parasitaires et établir le rôle de chacune d'elle dans la propagation des allèles impliqués dans la résistance à l'artémisinine.

Impact de séquençage

Le stage d'observation à Paris en octobre 2012 m'a permis d'être formé à la technique de séquençage à haut débit (NGS) et m'a donné l'occasion de mettre en route le séquençage de 25 génomes. Ce travail a été effectué sous la direction de Mme Christiane Bouchier à la plateforme Génomique, à la Génopôle, Institut Pasteur à Paris. Mes différents séjours à Montpellier (novembre-décembre 2012, septembre-octobre 2013 et septembre-octobre 2014) m'ont permis d'intégrer les données du barcode avec les données de génomiques disponible (MalariaGEN et PlasmoDB). Dans le travail mené par Ariey et collaborateurs (Ariey et al. 2014), l'approche de séquençage du génome complet à haut débit des souches 3D7, F32-TEM, F32-ART5, de 21 souches cambodgiennes a permis de définir un marqueur moléculaire sélectif associé à la résistance à l'artémisinine. Le travail de séquençage a été poursuivi jusqu'à obtenir maintenant près de 50 souches séquencées. Ces valeurs contiennent une grande quantité de données qui doit maintenant être exploitée. Un énorme effort de séquençage a aussi été mené du côté du Wellcome Trust avec près de 300 souches séquencées au Cambodge. Une collaboration devra être établie avec le consortium MalagiaGEN pour intégrer l'ensemble de ces données. Ceci devrait nous permettre dans un premier temps de

développer un système de typage des isolats cliniques par sous-population et par caractérisation de l'allèle Kelch présent. Nous souhaitons dans un deuxième temps pouvoir comprendre quelle sont les propriétés particulière des sous-populations de parasites. Nous allons étudier la distribution de ces allèles dans les réseaux métabolique et regarder aussi les liens entre ces allèles et les termes d'annotation de la Gene Ontology. Nous espérons ainsi trouver de nouvelles pistes pour comprendre la structuration si particulière des sous-populations dans l'ouest du Cambodge favorisant l'émergence de résistances puis leur diffusion dans le monde entier. La maitrise de la diffusion des résistances aux dérivés de l'artémisinine est en enjeu de santé publique majeur pour les années à venir.

Le travail de thèse que j'ai réalisé a permis de proposer de nouveaux outils plus performants pour améliorer notre connaissance de l'épidémiologie du paludisme au Cambodge et sa dynamique et/ou son adaptation au cours du temps et d'évaluer l'impact des stratégies mise en œuvre par le Programme National de lutte contre le Paludisme au Cambodge.

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