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

Running title: In-vitro and ex-vivo detection of artemisinin resistance

Benoit Witkowski, Chanaki Amaratunga, Nimol Khim, Sokunthea Sreng, Pheaktra Chim, Saorin Kim, Pharath Lim, Sivanna Mao, Chantha Sopha, Baramey Sam, Jennifer M. Anderson, Socheat Duong, Char Meng Chuor, Walter R. J. Taylor, Seila Suon, Odile Mercereau-Puijalon, Rick M. Fairhurst, Didier Menard

Malaria Molecular Epidemiology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia
B Witkowski, PhD
N Khim, MSc
P Chim
S Kim
P Lim, MD
D Menard, PhD

Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA
C Amaratunga, PhD
P Lim, MD
JM Anderson, PhD
RM Fairhurst, MD

National Center for Parasitology, Entomology and Malaria Control, Phnom Penh, Cambodia
S Sreng
P Lim, MD
S Suon, MD
Prof S Duong, MD
CM Chuor, MD

Sampov Meas Referral Hospital, Pursat, Cambodia
S Mao, MD

Makara 16 Referral Hospital, Preah Vihear, Cambodia
C Sopha, MD

Ratanakiri Referral Hospital, Ratanakiri, Cambodia
B Sam, MD

Service de Médecine Internationale et Humanitaire, Hôpitaux Universitaires de Genève, Geneva, Switzerland
WRJ Taylor, MD

Parasite Molecular Immunology Unit, Institute Pasteur, Paris, France
O Mercereau-Puijalon, PhD

Co-first authors:

Benoit Witkowski
Chanaki Amaratunga

Co-corresponding authors:

Dr. Didier Menard
Institut Pasteur du Cambodge
5 Boulevard Monivong – BP 983
Phnom Penh, Cambodia
dmenard@pasteur-kh.org

Dr. Rick M. Fairhurst
National Institutes of Health
12735 Twinbrook Parkway, Room 3E-10A
Rockville, MD 20852, USA
rfairhurst@niaid.nih.gov

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Abstract

Background. Artemisinin resistance in *Plasmodium falciparum* has been documented in Cambodia, Thailand, Myanmar, and Vietnam. This clinical phenotype manifests as a long parasite clearance half-life during administration of artemisinin monotherapy or artemisinin-based combination therapy. The lack of in-vitro and ex-vivo correlates of artemisinin resistance makes studying this phenotype costly and logistically challenging.

Methods. We culture-adapted parasites from patients with long and short parasite clearance half-lives from a study conducted in Pursat, Cambodia, in 2010 and used novel in-vitro survival assays to explore the stage-dependent susceptibility of slow- and fast-clearing parasites to dihydroartemisinin. In 2012, we implemented the Ring-stage Survival Assay (RSA) in prospective parasite clearance studies in Pursat, Preah Vihear, and Ratanakiri, Cambodia, to measure the ex-vivo responses of parasites from patients with malaria. The clinical studies are registered with ClinicalTrials.gov, numbers NCT00341003 and NCT01736319.

Findings. In-vitro survival rates of culture-adapted parasites from 13 slow- and 13 fast-clearing infections differed significantly when assays were conducted on 0-3 h ring-stage parasites (RSA^{0-3h}, 10·9% 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 to 0·87; p<0·0001).

Interpretation. We report for the first time in-vitro and ex-vivo assays that detect artemisinin-resistant *P falciparum* in Cambodia. The in-vitro RSA^{0-3h} provides a platform for biochemical and molecular characterization of artemisinin resistance. The ex-vivo RSA can be easily implemented in countries where surveillance for artemisinin resistance is needed.

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Keywords: *Plasmodium falciparum*, malaria, artemisinin resistance, parasite clearance half-life, in-vitro testing, ex-vivo testing, Cambodia

Introduction

Following the World Health Organization's recommendation[1] to use artemisinin-based combination therapies (ACTs) for *Plasmodium falciparum* malaria, the burden of this disease fell substantially.[2] As with earlier antimalarial drugs,[3] parasite resistance to artemisinin and its derivatives has emerged in southeast Asia. Since first being reported in 2008 and 2009 from Battambang[4] and Pailin[5] provinces in western Cambodia, artemisinin-resistant *P falciparum* malaria has been documented elsewhere in western Cambodia,[6,7] western Thailand,[8] southern Myanmar,[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 novel artemisinin resistance foci and implementation of containment interventions are a top priority.[13] Although artemisinin resistance has not been precisely defined, it is presently recognized 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 ≥10,000 per μL of blood (ie, ≥0·2% parasitemia).[15] In areas of low malaria transmission like Cambodia, parasite clearance studies require screening thousands of febrile individuals over entire transmission seasons to enroll few (<5%) patients who meet inclusion criteria and agree to several days of hospitalization. Such studies are thus logistically and financially demanding, as well as inconvenient for patients and their families. There is thus 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) may 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) may be useful in mapping the geographical spread or worsening of

artemisinin resistance in real-time, thus providing actionable information for national malaria control programs. To date, 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 demonstrated.[4-6] One potential reason for this observation is that parasites in these assays are exposed to very low concentrations of dihydroartemisinin (DHA, the active metabolite of all artemisinins) for 48-72 h, while parasites in vivo are exposed to much higher concentrations of DHA 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] (figure 1A), we recently measured the susceptibility of 0-12 h post-invasion rings to a pharmacologically-relevant exposure (700 nM for 6 h) to DHA. We observed a 17-fold higher survival rate of culture-adapted parasite isolates from Pailin province, an area of artemisinin resistance in western Cambodia, compared to those from Ratanakiri province, an area of artemisinin sensitivity in eastern Cambodia. How this geographic dichotomy relates to the clinical artemisinin resistance phenotype is unknown, as ring-stage parasites from patients with known parasite clearance kinetics have not yet been tested in the RSA.

The objectives of the present study were: (i) to assess whether an in-vitro RSA can distinguish culture-adapted *P falciparum* isolates from patients with slow- or fast-clearing infections; (ii) to investigate the stage-dependent susceptibility of parasites to DHA in the in-vitro RSA, and (iii) 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 DHA, we exposed synchronized, ring-stage parasites to brief, high-dose pulses of this drug. In a retrospective study using culture-adapted parasites collected in 2010,[6] we demonstrate increased survival of 0-3 h

ring-stage parasites from slow-clearing compared to fast-clearing infections. In a prospective study, we show that ex-vivo parasite survival rates correlate significantly with in-vivo parasite clearance half-lives, and reliably detect artemisinin-resistant parasites in patients. These data identify the artemisinin-resistant developmental stage of clinical *P falciparum* isolates, and provide in-vitro and ex-vivo phenotypes for investigating the molecular basis and geographic distribution of artemisinin resistance.

Methods

Study design, patients, and drug therapy

We conducted two clinical studies in Cambodia to measure therapeutic responses to artesunate: in 2009-2010 in Pursat province (western Cambodia),[6] where artemisinins have been used for 35 years and artemisinin resistance is well established; and in 2012 in Pursat province and also in Preah Vihear (northern Cambodia) and Ratanakiri provinces (eastern Cambodia), where ACTs were first used in 2000 and artemisinin resistance has not yet been reported. The studies were conducted in referral hospitals in each province. The Cambodian National Ethics Committee for Health Research and the U.S. National Institute of Allergy and Infectious Diseases Institutional Review Board approved both studies.

The 2009-2010 study in Pursat was previously reported.[6] Patients were treated with oral doses of 4 mg/kg artesunate at 0, 24, 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 over 1 year of age and non-pregnant adults with uncomplicated falciparum malaria (parasite density ≥10,000 and ≤200,000 per µL of blood) were enrolled if written informed consent was obtained from the patient or, if a child, from a parent or guardian. Patients with severe malaria, *P vivax* infection, hematocrit <25%, antimalarial drug use in the last 7 days, or known allergy to artemisinins or piperaquine were excluded. Patients were treated with oral doses of Duo-Cotecxin®

(containing 40 mg DHA and 320 mg piperaquine per tablet; Holleypharma, China) at 0, 24, and 48 h. The doses were based on body weight as follows: 0·5 tablet (<10 kg), 1 tablet (10-19 kg), 1·5 tablets (20-29 kg), 2 tablets (30-39 kg), and 3 tablets (≥40 kg).

Parasite density count, staging, and clearance

In the 2009-2010 study, thick blood films were made from patients prior to the first dose of artesunate (0 h) and then every 6 h until asexual parasitemia was undetectable.[6] In the 2012 study, blood films were made at 0, 2, 4, 6, 8, and 12 h, and then every 6 h until parasitemia was undetectable. Parasite developmental stages at 0 h were estimated as tiny or large rings based on 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 using the parasite clearance estimator (https://www.wwarn.org/research/parasite-clearance-estimator).[15] The half-life was considered interpretable when the R^2 value of the slope regression line was > 0.8.

In-vitro parasite adaptation

In the 2009-2010 study, blood samples were collected into ACD vacutainers (Becton-Dickinson, Franklin Lakes, NJ) at 0 h. Parasitized erythrocytes were cryopreserved in Glycerolyte 57 (Baxter Healthcare Corp., IL)[21] immediately or after short-term cultivation, and stored in liquid nitrogen until use. From 89 patients enrolled, we selected 18 fast- 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 performed on parasites from 13 fast- 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).

Isotopic in-vitro sensitivity testing (ISA)

The in-vitro sensitivity of culture-adapted parasites to artesunate and DHA (obtained from the Worldwide Antimalarial Resistance Network, WWARN) was assessed using a 48-h isotopic test[20] with drug concentrations ranging from 0·1-102·4 nM for artesunate, and 0·0625-64 nM for DHA. The quality of in-vitro assays was monitored using 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 to drug-free controls. IC_{50} and IC_{90} values were determined by non-linear regression using ICEstimator software (www.antimalarial-icestimator.net).[22,23]

In-vitro survival assays

Culture-adapted parasites were synchronized twice using 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% hematocrit and 1% parasitemia by adding uninfected erythrocytes, and dispensed (2 mL/well in a 24-well culture plate) into two parallel cultures. The RSA^{0-3h} was performed immediately with 0-3 h post-invasion rings, the RSA^{9-12h} with 9-12 h post-invasion rings, and the Trophozoite-stage Survival Assay (TSA^{18-21h}) with 18-21 h post-invasion trophozoites (figure 1A).

In each assay, parasites were exposed to 700 nM DHA or 0·1% DMSO 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^{0-3h}), 57 h (RSA^{9-12h}), and 48 h (TSA^{18-21h}) after drug removal. For each sample, approximately 10,000 erythrocytes were examined independently by two microscopists who were blinded to each other's data and to half-lives. When the difference between survival rates was >20%, a third blinded microscopist examined the slides.[24] Mean parasite counts were calculated and survival rates expressed as ratios of viable parasitemias in DHA- and DMSO-exposed samples.

Ex-vivo survival assay

In the 2012 study, ex-vivo RSAs were performed on parasites obtained directly from patients (figure 1B). Two mL of venous blood were collected into ACD vacutainers (Becton-Dickinson) prior to the first Duo-Cotecxin® dose and processed within 24 h. Plasma was removed and the blood washed three times in RPMI-1640. If the parasitemia was >1%, it was adjusted to 1% by adding uninfected erythrocytes. Ex-vivo RSAs were performed as above except that complete medium did not contain human plasma, parasites were not experimentally synchronized, and three different atmospheres were tested in parallel: tri-gas, 5% CO_2, and candle jar. Smears made 66 h after drug removal were examined and survival rates calculated as described above. Results were considered interpretable if the parasitemia in the DMSO-exposed sample was higher than the starting parasitemia.

Parasite genotyping

DNA was extracted from 200 µL of whole blood collected in 2010 at 0 h and from corresponding culture-adapted parasites just prior to in-vitro assays using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). Parasite genotyping was performed as described.[25] Twelve single nucleotide polymorphisms were assessed using a PCR-LDR-FMA (Polymerase Chain Reaction-Ligase Detection Reaction-Fluorescence Microspheres Assay) (appendix).

Statistical analysis

Data were analyzed using Microsoft Excel and MedCalc version 12 (Mariakerke, Belgium). Quantitative data were expressed as median (interquartile range, IQR). Stage-dependent patterns of survival were expressed as the difference between $RSA^{0\text{-}3h}$ and $TSA^{18\text{-}21h}$ (Δ). Continuous variables were compared using Mann-Whitney *U* test. Correlations were analyzed using Spearman Correlation test. Ex-vivo RSA values that were obtained in three atmospheric conditions were compared using One-way Repeated-measures ANOVA with Bonferroni correction for p-values (Friedman test). A p-value <0·05 was deemed significant.

Role of the funding source

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

Results

We culture-adapted parasites from 13 fast- and 13 slow-clearing infections collected in Pursat in 2010 (appendix), and used them in three stage-specific survival assays: RSA^{0-3h}, RSA^{9-12h}, and TSA^{18-21h} (figure 1A). For 0-3 h rings (RSA^{0-3h}), the median survival rate of slow-clearing parasites was 55-fold higher than that of fast-clearing parasites (10·9% vs. 0·23%; p=0·007) (table; figure 2). In contrast, 9-12 h rings and 18-21 h trophozoites from fast- and slow-clearing infections showed no difference in survival (table; figure 2). The stage-dependent survival patterns differed between fast- and slow-clearing parasites (appendix). Specifically, the survival rates of slow-clearing parasites decreased with parasite stage, while those of fast-clearing parasites increased with parasite stage (Δ=9·9%, IQR 1·7%-14·4% vs. Δ= -0·3%, IQR -1·1%-0·4%; p=0·007). In an isotope-based sensitivity assay (ISA) that monitored replication of parasites exposed to drug for 48 h,[26] fast- and slow-clearing parasites did not differ significantly in IC_{50} and IC_{90} values for artesunate or DHA (table; appendix).

In patients with falciparum malaria, the age distribution of circulating ring-stage parasites is heterogeneous, ranging 0-18 h at the time of clinical presentation[27]; that is, ring-stage parasites are not necessarily tightly synchronized at the 0-3 h age of development. We therefore sought to investigate whether an ex-vivo RSA could distinguish fast- from slow-clearing parasites that have been neither culture-adapted nor experimentally synchronized. In a prospective study, we obtained parasites directly from consecutively-enrolled patients in Pursat, Preah Vihear, and Ratanakiri in 2012, and exposed them to 700 nM DHA for 6 h in three different atmospheres. 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 may not be available or affordable. Eighty-three per cent (30/36) of patients had interpretable half-life values and tri-gas survival rates (appendix), which correlated significantly (r=0·74, 95% CI 0·50-0·87; p<0·0001) (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, while the other six parasites with a median 0·70% survival rate (IQR 0·18-2·0) had a median 2·28-h half-life (IQR 1·89-3·52). In Ratanakiri, one parasite with a 38·3% survival rate had a 9·06-h half-life, while the other ten parasites had a median 0·40% survival rate (IQR 0·26-1·48) and a median 2·28-h half-life (IQR 1·90-2·64). These findings suggest that artemisinin-resistant *P falciparum* has spread or independently emerged in northern and eastern Cambodia, a possibility that can now be confirmed using the in-vitro RSA^{0-3h}.

Discussion

Here we show that *P falciparum* isolates from slow- and fast-clearing infections in Cambodia respond differently to a 6-h, 700-nM exposure to DHA. In the RSA^{0-3h}, the 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 DHA susceptibility data that correlate with in-vivo parasite clearance half-lives. These data qualify the in-vitro RSA^{0-3h} as a novel laboratory tool for elucidating the mechanism of artemisinin resistance through biochemical and molecular studies. These may include: genome-wide association studies in which RSA^{0-3h} survival rates are associated with whole-genome SNP data;[28] phenotypic screening of parasite progeny clones obtained from genetic crosses between artemisinin-sensitive and artemisinin-resistant parental lines; phenotypic characterization of

the different artemisinin-resistant parasite subpopulations circulating in western Cambodia;[28] and validation of candidate molecular markers through genetic manipulation of parasites.

The data also indicate 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 in areas where it is entrenched (eg, western Cambodia). Both types of findings from ex-vivo RSAs may inform national malaria control programs to expand or intensify containment measures. In a "screen-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 DHA resistance in this assay can then be (i) adapted to short-term culture in the laboratory, (ii) genotyped to ensure its identity to the clinical parasite isolate obtained from the patient, and (iii) tested to "confirm" DHA resistance in the in-vitro RSA^{0-3h}.

For both artemisinin-sensitive and artemisinin-resistant parasites, we demonstrate stage-dependent heterogeneity of DHA susceptibility in ring forms. In artemisinin-sensitive parasites, 0-3 h rings were more susceptible to DHA than 9-12 h rings. This finding with clinical parasite isolates is consistent with the recent observation that 2-4 h rings of artemisinin-sensitive laboratory lines are specifically hypersensitive to DHA.[18] In artemisinin-resistant parasites, on the other hand, 0-3 h rings were less susceptible to DHA than 9-12 h rings. We tentatively conclude that the susceptibility of Cambodian parasites to DHA is controlled predominantly at the 0-3 h stage of parasite development. This interpretation, and our finding that trophozoites are susceptible to DHA regardless of half-life, is consistent with mathematical modeling predictions[29] and transcriptomics data[30] from studies of ring-stage parasites.

Half-lives and RSA^{0-3h} 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 hypothesized that these three parasites had already developed into DHA-susceptible, late ring-stage parasites in the patient's blood at the time of the first artesunate dose.

To investigate this possibility, we reviewed the initial blood smears from these patients and estimated the relative age of their ring-stage parasites just prior to artesunate treatment (appendix). In thin blood smears made at 0 h, we found that these three 'discordant' patients indeed had a 2-fold lower proportion of tiny rings compared to the 12 'concordant' patients from the slow-clearing group (ie, those having slow-clearing infections with DHA-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 DHA than tiny, young rings. Overall, the data suggest that the relative abundance of tiny and large rings at the time of the first artemisinin dose influences the parasite clearance half-life, and that accurate ex-vivo staging of parasites is critical 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[31] or insufficient plasma levels of artemisinins.

The RSA^{0-3h} survival rate may be critically informative in ongoing parasite genetics studies[28,32,33] aimed at identifying loci under artemisinin selection since it is unaffected by in-vivo parameters (eg, pharmacokinetics, hemoglobin type, and acquired immunity) that may influence the parasite clearance half-life. While this phenotype may also be a useful readout in studies to define and validate molecular markers for tracking artemisinin-resistant parasites in the field, the RSA^{0-3h} is a laborious assay. In 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 alterations that may accompany the culture adaptation of parasites. In addition to implementing methodologies that more precisely determine the age of rings, FACS- or ELISA-based analysis of parasite viability should improve the throughput of DHA-susceptibility studies. Until such methods are developed and validated, we propose the simple ex-vivo RSA as a highly-informative surveillance tool for identifying artemisinin-resistant parasites in areas where slow parasite clearance is suspected. Investigating the relationship between RSA survival rates ex vivo and parasite recrudescence rates in vivo may be useful in assessing the clinical impact of artemisinin resistance.

Panel: Research in context

Systematic Review

P falciparum is capable of withstanding tremendous drug pressure by developing resistance or tolerance. Such phenotypic responses to drug treatments have been observed since the introduction of antimalarials, including the most recently introduced artemisinin (ART) class of drugs and their quinoline partners that comprise artemisinin-based combination therapies (ACTs). We searched PubMed with the terms ‘drug-resistant malaria’, limited our search to clinical trials, and used no date or language restrictions. This process produced 772 publications, the first of which reported on chloroquine-resistant *P falciparum* malaria. The World Health Organization currently recommends ACTs to treat falciparum malaria in all endemic countries. ART is a very potent antimalarial, initially described as being less prone to selecting resistant parasites. Adding the term ‘artemisinin’ to the search produced 119 reports. Since the first conclusive report of ART-resistant malaria from western Cambodia in 2008, there is clear evidence that ART resistance is emerging elsewhere.

The in-vivo phenotype of ART resistance is presently defined as a relatively long parasite clearance half-life following ACT. Only eight clinical trials that were designed to generate half-life data have been published to date.[6-10,34-36] Unlike with previous classes of antimalarials, in-vitro isotope-based sensitivity assays (ISAs) using artemisinins or their active metabolite dihydroartemisinin (DHA), do not significantly correlate with in-vivo efficacy results. Our own recent studies have shown that the format of in-vitro sensitivity testing for ART or DHA (continuous exposure to very low drug levels during the entire cycle of parasite development) do not adequately investigate the parasite stage-restricted phenotype of ART resistance or account for the particular pharmacodynamics of ARTs. Mathematical modeling of ART resistance has predicted that early ring-stage parasites express the ART resistance phenotype. Novel in-vitro assays have uncovered a peculiar early ring response to ARTs, such as better survival and ART-induced quiescence. However, these experimental observations were reported solely for long-term-adapted *P falciparum* lines, and in the absence of any clinical context.

Here we describe a novel assay, the Ring-stage Survival Assay (RSA), specifically designed to assess the ring-stage susceptibility of *P falciparum* to DHA. In a retrospective study using culture-adapted parasites, we demonstrate increased survival of 0-3 hour ring-stage parasites from slow-clearing compared to fast-clearing infections. In a prospective study, we show that ex-vivo parasite survival rates correlate significantly with in-vivo parasite clearance half-lives, and reliably detect artemisinin-resistant parasites in patients with malaria.

Interpretation

We report for the first time in-vitro and ex-vivo assays that detect artemisinin-resistant, slow-clearing *P falciparum* parasites in patients with malaria. The in-vitro RSA^{0-3h} provides a platform for biochemical and molecular characterization of artemisinin resistance. The ex-vivo RSA can be easily implemented in countries where surveillance for artemisinin resistance is needed.

Contributors

BW, CA, PL, JMA, SK, SD, CMC, WRT, O M-P, RMF, and DM contributed to study design. NK genotyped parasites. BW, CA, and PC performed in-vitro and ex-vivo drug assays. SSr, SM, CS, BS, and SSu gathered clinical data. BW, CA, OMP, RMF, and DM analysed data and wrote the manuscript.

Conflicts of interest

The authors have declared that no conflicting interests exist.

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Table 1. Results of in-vitro assays using culture-adapted parasite isolates.

Percentage survival in RSA^{0-3h}, RSA^{9-12h}, and RSA^{18-21h}, and IC_{50} and IC_{90} values for dihydroartemisinin (DHA) and artesunate (ATS) in isotope-based sensitivity assays. P-values for significance from Mann-Whitney test.

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

Figure legends

Figure 1. Schematic description of various dihydroartemisinin survival assays performed on parasite isolates.

Panel A shows the level of synchronization and timing of dihydroartemisinin (DHA) exposure for four in-vitro survival assays performed on culture-adapted *P falciparum* isolates: Ring-stage Survival Assay (RSA) previously described by Witkowski *et al.*,[20] RSA^{0-3h}, RSA^{9-12h}, and TSA^{18-21h}. 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). Due to 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.

Panel B shows the timing of DHA exposure for the ex-vivo survival assay performed on circulating, ring-stage parasites (0-18 h) obtained directly from the blood of patients with uncomplicated malaria: ex-vivo RSA. This assay thus measures the DHA susceptibility of the parasite isolate – at the same developmental stage and at the same time – as the in-vivo parasite clearance study.

Figure 2. Results of in-vitro dihydroartemisinin survival assays.

Results from the RSA^{0-3h} (pink), RSA^{9-12h} (dark blue), and TSA^{18-21h} (purple) are expressed as the percentage of viable *P falciparum* parasites following a 6-h exposure of 0-3 h rings, 9-12 h rings, and 18-21 h trophozoites to 700 nM dihydroartemisinin (DHA) compared to DMSO-exposed controls. These assays were performed on culture-adapted parasite isolates obtained from 13 patients with fast-clearing (filled circles) infections and 13 patients with slow-clearing infections (open circles) in Pursat in 2010.

The horizontal lines and I bars represent the medians and interquartile ranges. The solid and dotted grey lines represent the stage-dependent survival pattern of parasites from slow- and fast-clearing infections, respectively.

Figure 3. Correlation of in-vivo parasite clearance half-lives and ex-vivo dihydroartemisinin survival rates.

Ex-vivo Ring-stage Survival Assays (RSAs) were performed 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 percentage of viable parasites following a 6-h exposure to 700 nM dihydroartemisinin (DHA) compared to DMSO-exposed controls. Results from the parasite clearance studies are expressed as the parasite clearance half-life in hours. The percentage of viable parasites in ex-vivo RSAs correlated significantly with the parasite clearance half-life ($r=0{\cdot}74$, 95% CI 0·50-0·87; $p<0{\cdot}0001$) in Pursat (red), Preah Vihear (blue), and Ratanakiri (green).

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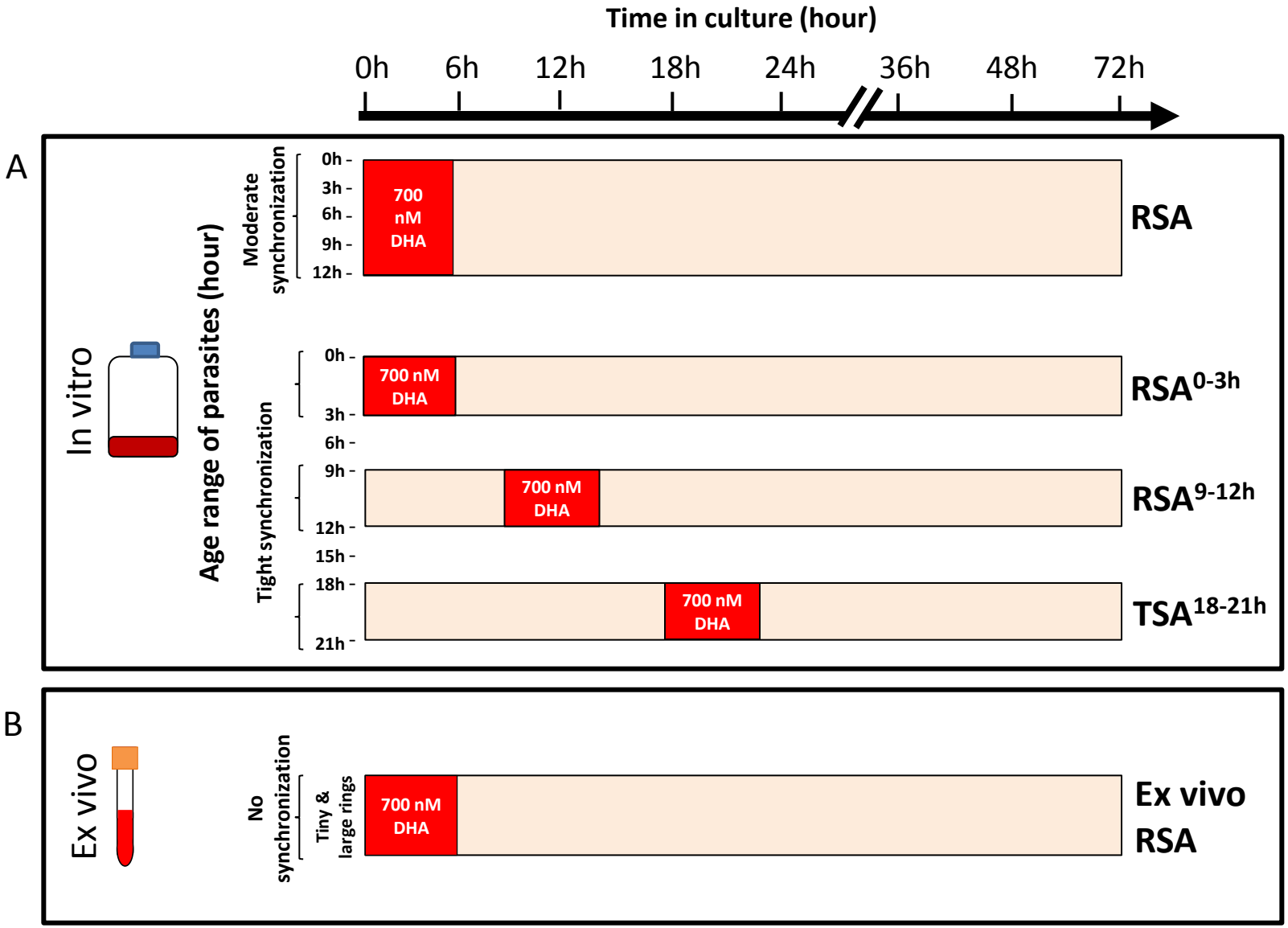
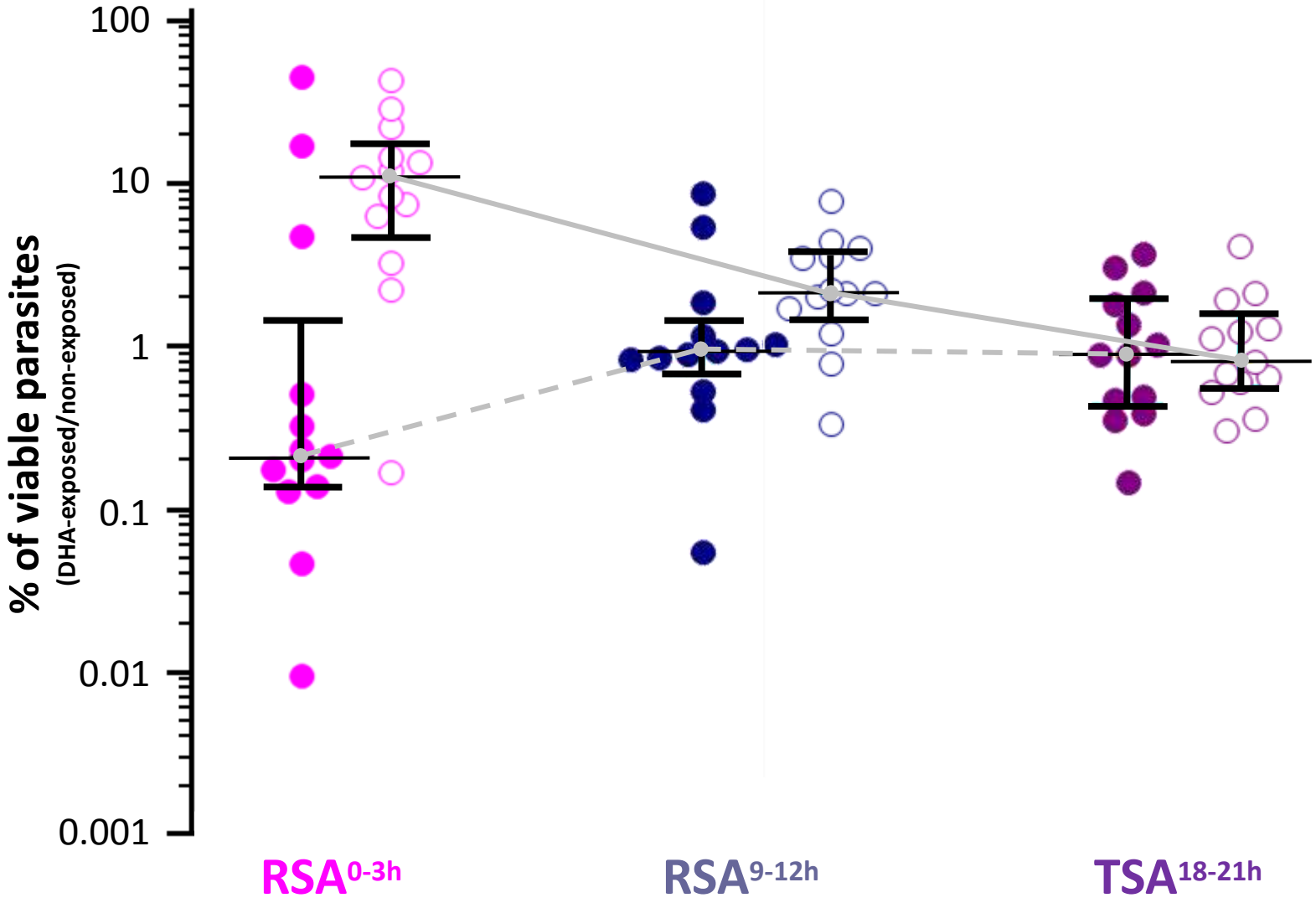
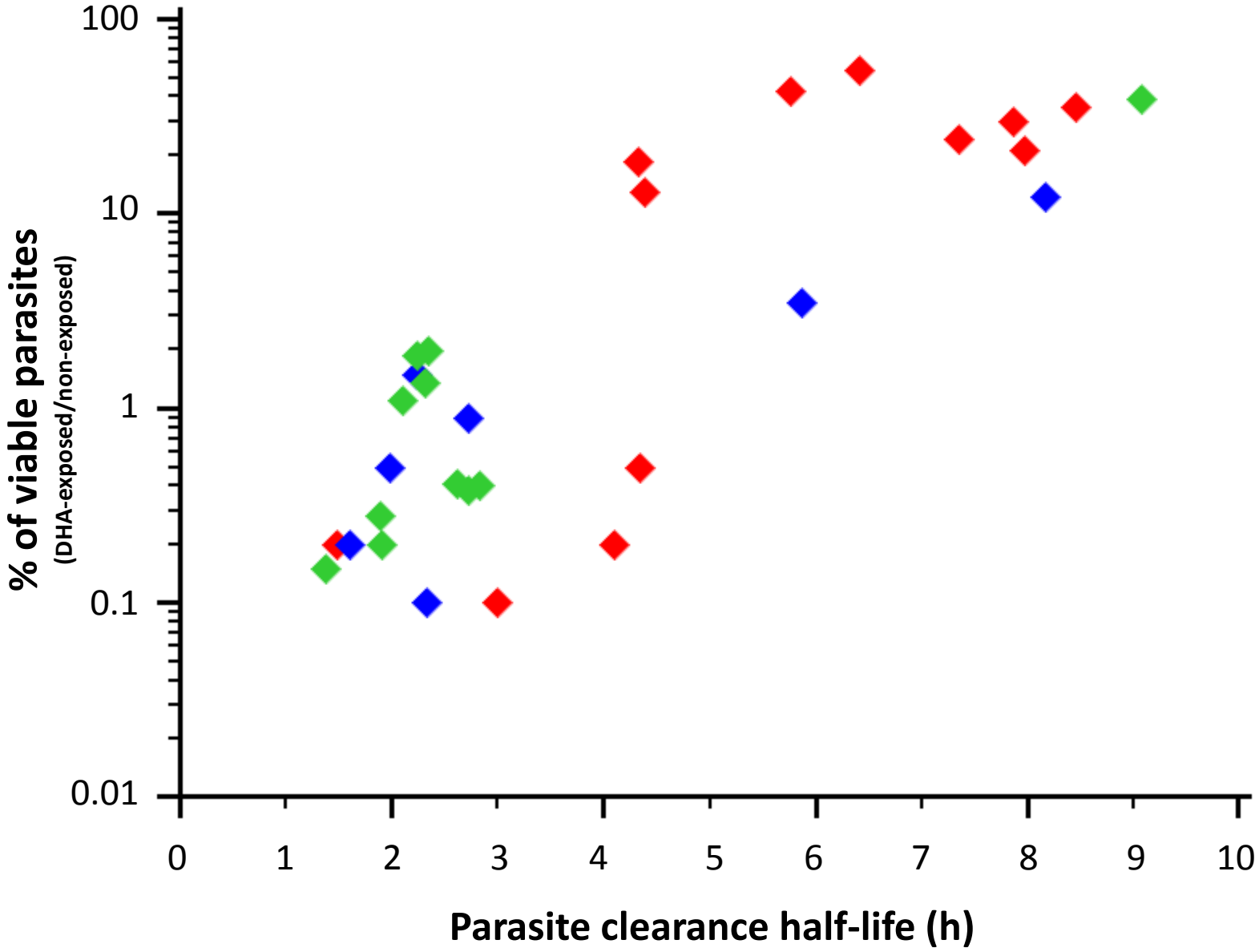

Time in culture (hour)
0h
6h
12h
18h
24h
36h
48h
72h
A
In vitro
Age range of parasites (hour)
Moderate synchronization
0h
3h
6h
9h
12h
700 nM DHA
RSA
Tight synchronization
0h
3h
700 nM DHA
RSA0-3h
6h
9h
12h
700 nM DHA
RSA9-12h
15h
18h
21h
700 nM DHA
TSA18-21h
B
Ex vivo
No synchronization
Tiny & large rings
700 nM DHA
Ex vivo RSA


100
10
1
0.1
0.01
0.001
% of viable parasites
(DHA-exposed/non-exposed)
RSA0-3h
RSA9-12h
TSA18-21h


100
10
1
0.1
0.01
% of viable parasites
(DHA-exposed/non-exposed)
0
1
2
3
4
5
6
7
8
9
10
Parasite clearance half-life (h)

APPENDIX MATERIAL

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

Benoit Witkowski, Chanaki Amaratunga, Nimol Khim, Sokunthea Sreng, Pheaktra Chim, Saorin Kim, Pharath Lim, Sivanna Mao, Chantha Sopha, Baramey Sam, Jennifer M. Anderson, Socheat Duong, Char Meng Chuor, Walter R. J. Taylor, Seila Suon, Odile Mercereau-Puijalon, Rick M. Fairhurst, Didier Menard

Contents

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^{0-3h} survival rate (%)	RSA^{9-12h} survival rate (%)	TSA^{18-21h} 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	aactttctctctattcttatttAAGGAGATAGTGTTGGGGGT	
8	CGAATTTAAGTACCTTAGGAAA	TCACAACGTCCATATGTTGAA	G	tcatcacttttctttacttttacattTGATGAAAGCCACCGAACTC	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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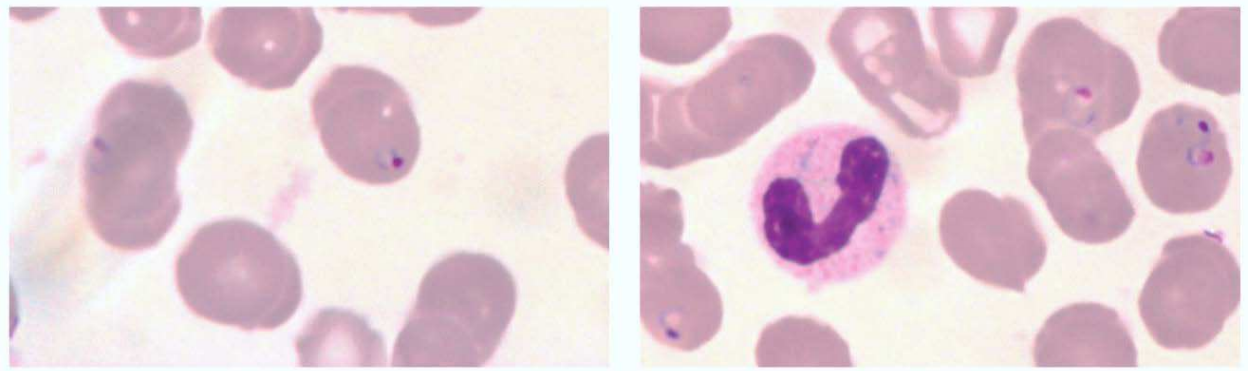
Appendix 3: Patient information and corresponding data from ex-vivo assays performed on *P. falciparum* isolates from Pursat, Preah Vihear, and Ratanakiri in 2012.

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

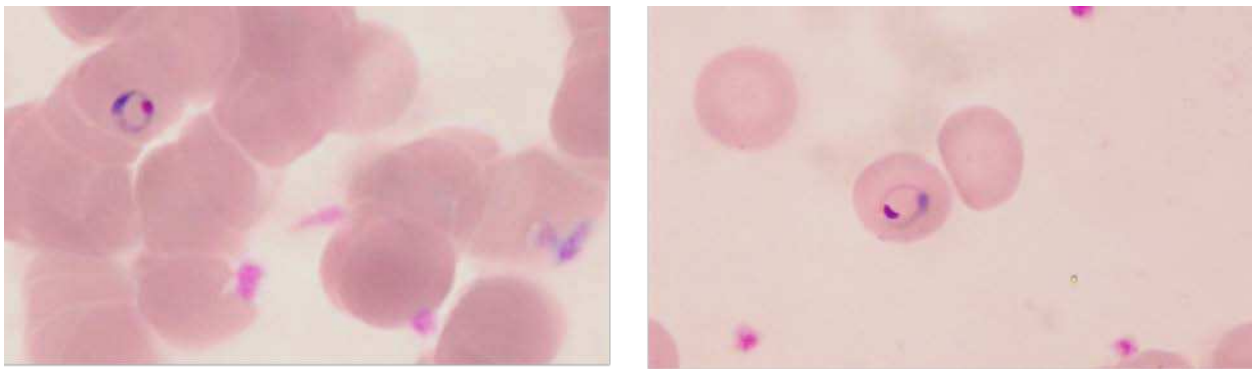
[1]Not done
[2]Not interpretable
KH1, KH2, and KH3 are identifying codes for Pursat, Preah Vihear, and Ratanakiri, respectively; these codes are *not* related to the parasite subpopulations reported by Miotto et al. (*Nat Genet*, 2013).

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

Panel A

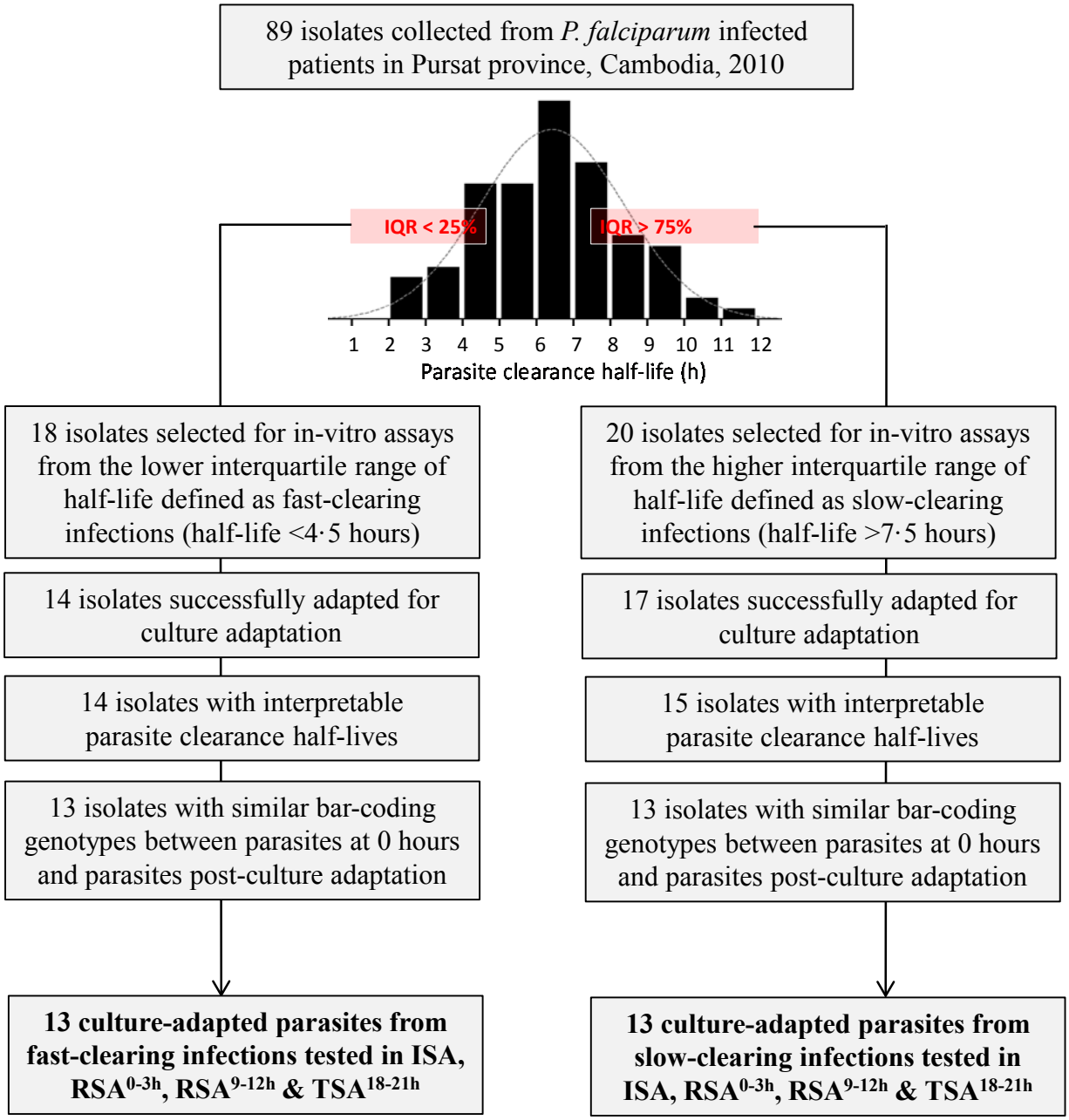


Panel B



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).

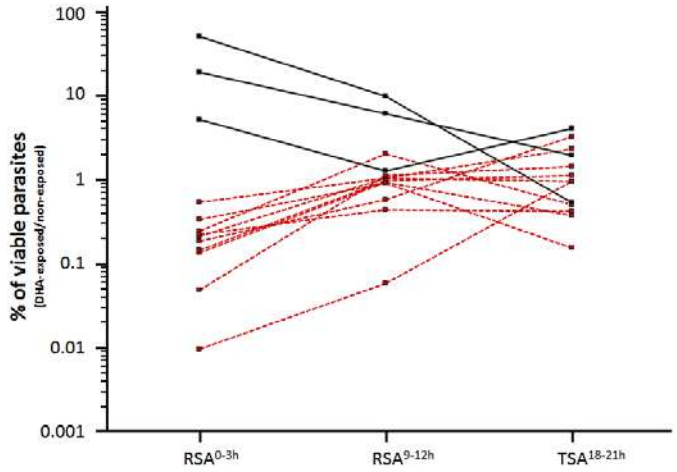
Appendix 5: Selection of *P falciparum* isolates from Pursat 2010 for culture adaptation and use in in-vitro assays.



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

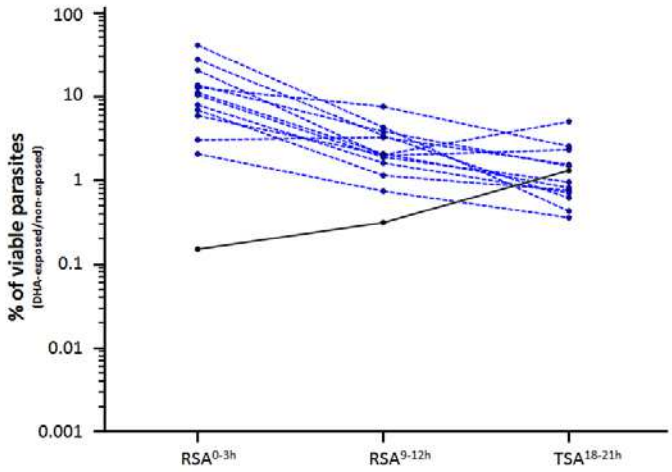
Appendix 6: Individual stage-dependent patterns in in-vitro survival assays (RSA^{0-3h}, RSA^{9-12h}, and TSA^{18-21h}) performed on parasite isolates from fast- (Panel A) and slow-clearing (Panel B) infections in Pursat in 2010.

Panel A



The dotted red lines represent the stage-dependent survival patterns of parasites that show 'concordance' between half-lives and RSA^{0-3h} 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^{0-3h} 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^{0-3h} 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^{0-3h} survival rate (Δ= -1·2%).