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Supplementary webappendix

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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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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	aactttctctctcattcttatttAAGGAGATAGTGTTGGGGGT	
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.

1. Barnadas C, Kent D, Timinao L, et al. A new high-throughput method for simultaneous detection of drug resistance associated mutations in *Plasmodium vivax dhfr, dhps* and *mdr-1* genes. *Malar J* 2011;**10**:282.
2. Carnevale EP, Kouri D, DaRe JT, McNamara DT, Mueller I, Zimmerman PA. A multiplex ligase detection reaction-fluorescent microsphere assay for simultaneous detection of single nucleotide polymorphisms associated with *Plasmodium falciparum* drug resistance. *J Clin Microbiol* 2007;**45**:752-61.
3. McNamara DT, Thomson JM, Kasehagen LJ, Zimmerman PA. Development of a multiplex PCR-ligase detection reaction assay for diagnosis of infection by the four parasite species causing malaria in humans. *J Clin Microbiol* 2004;**42**:2403-10.

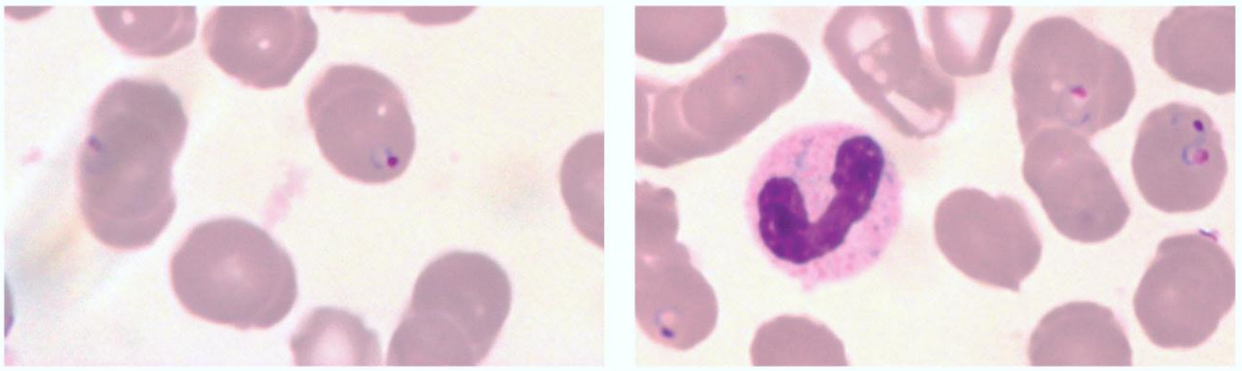
Appendix 3: Patient information and corresponding data from ex-vivo assays performed on *P. falciparum* isolates from Pursat, Preah Vihear, and Ratanakiri in 2012.

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

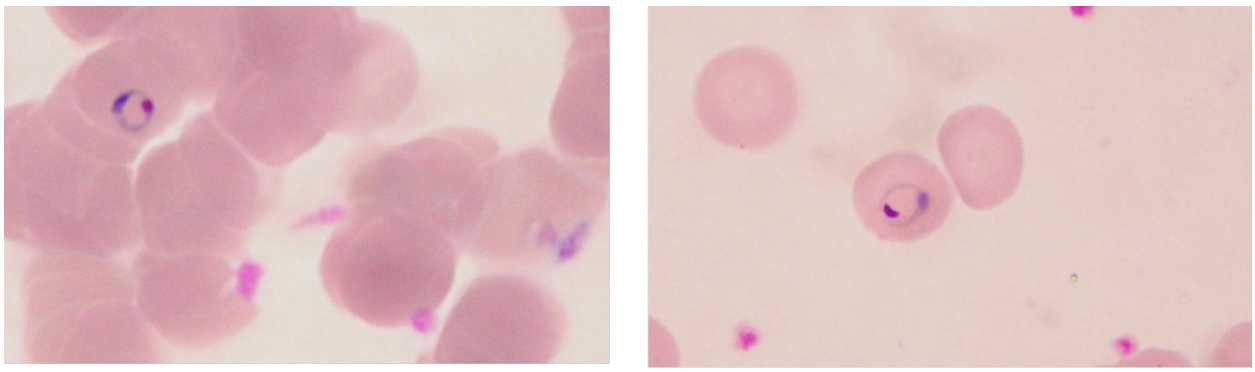
[1]Not done
[2]Not interpretable
KH1, KH2, and KH3 are identifying codes for Pursat, Preah Vihear, and Ratanakiri, respectively; these codes are *not* related to the parasite subpopulations reported by Miotto et al. (*Nat Genet*, 2013).

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

Panel A

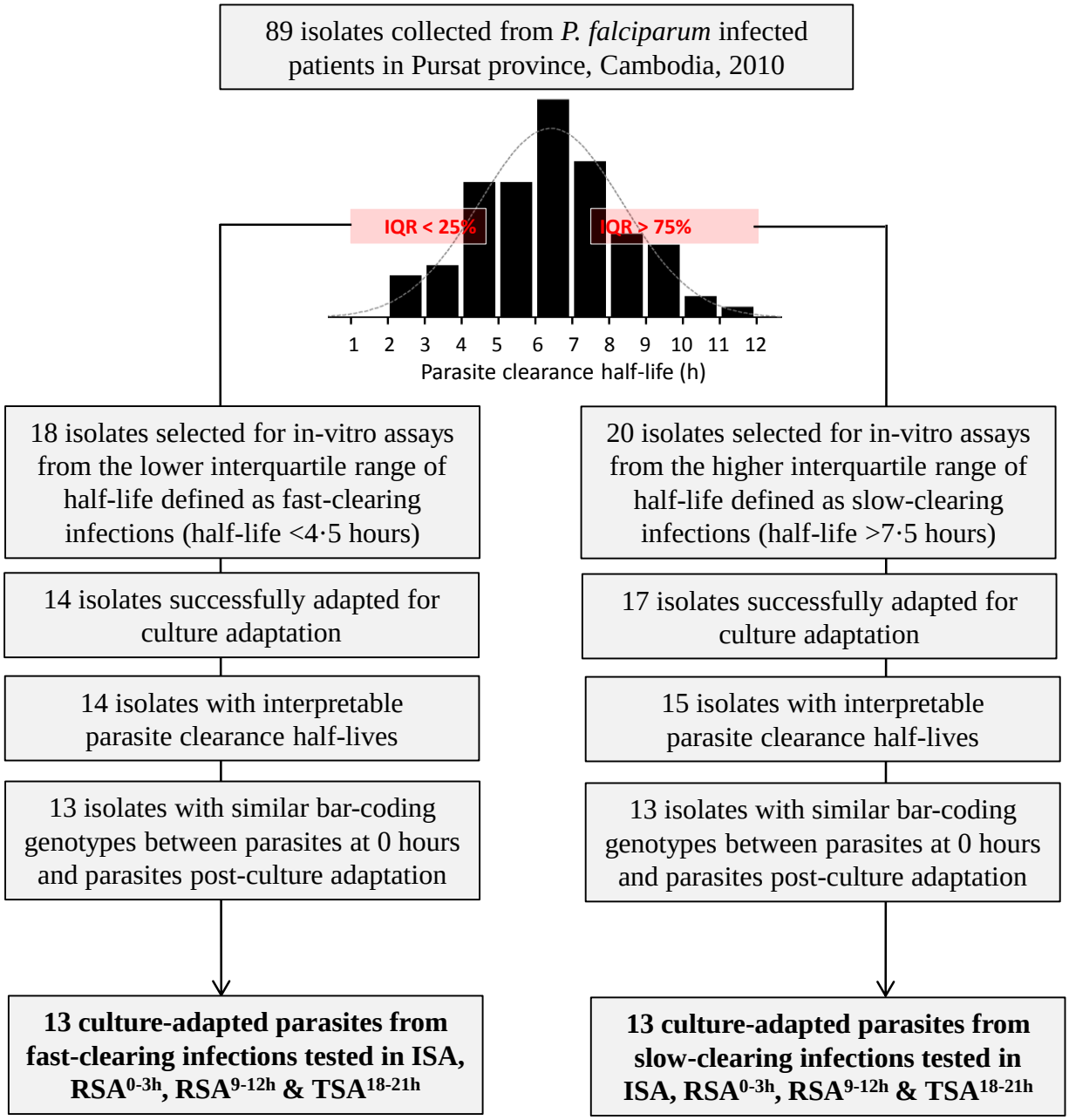


Panel B



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).

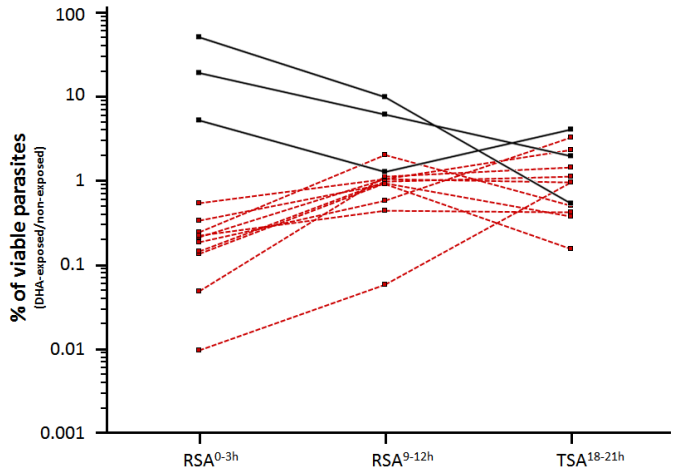
Appendix 5: Selection of *P falciparum* isolates from Pursat 2010 for culture adaptation and use in in-vitro assays.



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.

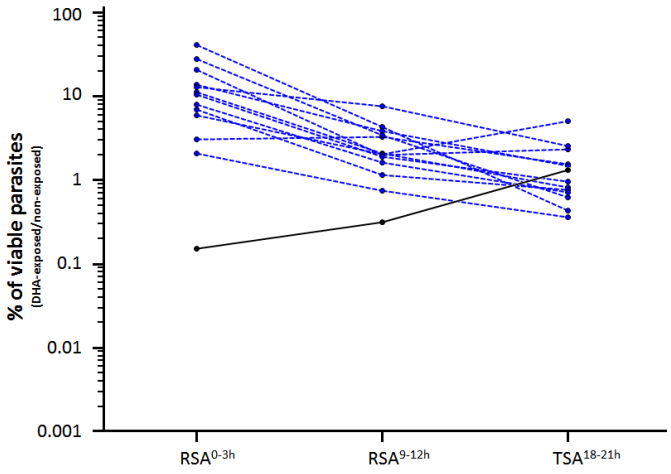
Appendix 6: Individual stage-dependent patterns in in-vitro survival assays (RSA^{0-3h}, RSA^{9-12h}, and TSA^{18-21h}) performed on parasite isolates from fast- (Panel A) and slow-clearing (Panel B) infections in Pursat in 2010.

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%).