Appendix 1

A list of reagents

PROCEDURE	REAGENT	SUPPLIER
PARASITE CULTURE	RPMI 1640	GIBCO
	HEPES BUFFER	GIBCO
	FOLIC ACID	SIGMA
	PABA	SIGMA
	GENTAMICIN SULPHATE	GIBCO
	SODUM BICARBONATE	SIGMA
	GAS(3% CO_2, 5% O_2, 92%N_2)	BOC(K)LTD
	SERUM	NON IMMUNE DONORS, NAIROBI, KENYA
	O POSITIVE RED BLOOD CELLS	BLOOD BANK, NAIROBI.
PARASITE STAINING	GIEMSA SOLUTION	BDH
	METHANOL	BDH
	IMMERSION OIL	BDH
SAMPLE COLLECTION	TRISODIUM CITRATE	BDH
	BOVINE ALBUMIN	SIGMA
CRYOPRESERVATION	GLYCEROL	SIGMA
	SODIUM CHLORIDE	SIGMA
	SORBITOL	SIGMA
	LIQUID NITROGEN	BOC (K) LIMITED
PARASITE STAINING	GIEMSA SOLUTION	SIGMA
	METHANOL	SIGMA
CHEMOSENSITIVITY TESTING	HCL	BDH
	CHLOROQUINE	SIGMA
	AMODIAQUINE	SIGMA
	PIPERAQUINE	DR STEVE WARD'S LABORATORY, LIVERPOOL
	DESETHYL AMODIAQUINE	SIGMA
	DIHYDROARTEMISININ	SIGMA
	SCINTILLATION COCKTAIL	PERKIN ELMER
GENOTYPING AND SEQUENCING	MAGNESIUM CHLORIDE	SIGMA
	dNTPS	FERMENTAS
	TAQ POLYMERASE	FERMENTAS
	1X PCR BUFFER	FERMENTAS
	ECoRI(RESTRICTION ENZYME)	NEW ENGLAND BIOLABS, BEVERLY, MA
	BsRI (RESTRICTION ENZYME)	NEW ENGLAND BIOLABS, BEVERLY, MA
	RESTRICTION BUFFER	NEW ENGLAND BIOLABS
	AFL III(RESTRICTION ENZYME)	NEW ENGLAND BIOLABS
	APOI (RESTRICTION ENZYME)	NEW ENGLAND BIOLABS
ANALYSIS OF PFMDR1 COPY NUMBER	TAQMAN PROBES	MWG BIOTECH
	TAQMAN BUFFER	MWG BIOTECH
RNA EXTRACTION	TRIZOL LS	INVITROGEN LIFE

		TECHNOLOGIES
	CHLOROFORM	BDH
	ISOPROPANOL	BDH
	ETHANOL	BDH
	DEPC H_20	AMBION
	3M NaOAc	BDH
	AGILENT RNA 6000 NANO KIT	AGILENT TECHNOLOGIES
CDNA SYNTHESIS	REVERSE TRANSCRPTASE	BIOSCRIPT ™
	OLIGO DT_{16}	BIOSCRIPT™
	RNASE FREE DNASE1	AMBION®
MICROARRAY HYBRIDIZATION	GENE CHIP-T7 OLIGOdT PROMOTER PRIME KIT	AFFYMETRIX
	SUPERSCRIPT II	INVITROGEN LIFE TECHNOLOGIES
	GENE CHIP SAMPLE CLEANUP MODULE	AFFYMETRIX
	10X TBE	CAMBREX
	DEPC TREATED WATER	AMBION
SYBR GREEN QPCR ASSAYS	SYBR GREEN PCR MASTERMIX	MWG BIOTECH
MYCOPLASMA TESTING	BIOTAQ™ CORE KIT 500IU	BIOLINE
	ATCC POSITIVE CONTROL KIT *(M.PIRUM, A. LAIDLAWAII)*	ATCC, MANASSAS, VA

A List of consumables

CONSUMABLES	**SUPPLIER**
NITROCELLULOSE FILTERS	PALL LIFE SCIENCES, MI, USA
VACUTAINERS	SARSTEDT
SYRINGES	SARSTEDT, UK
NEEDLES	SARSTEDT, UK
CRYOVIALS	NUNC, DENMARK
CENTRIFUGE TUBES	FALCON
PIPPETTES	FARENHEIT
MICROSCOPE SLIDES	SARSTEDT, UK
96 WELL FLAT BOTTOMED MICROCULTURE PLATES	COSTAR
PIPETTE TIPS	SARSTEDT, UK
GLASS FIBRE FILTER MATS	PERKIN ELMER
200uL PCR TUBES	INVITROGEN
MICROAMP 96 WELL PLATES	APPLIED BIOSYSTEMS
CULTURE FLASKS	CORNING
EPPENDORF TUBES	SARSTEDT, UK
GLOVES	TG SDN MALAYSIA
RNASE FREE TUBES	VWR SCIENTIFIC

Appendix 2

Primer sequences: mycoplasma testing

Primary PCR	**Secondary PCR**
1a- 5'ACACCATGGGAGYTGGTAAT3'	2e-5'GTGSGGMTGGATCACCTCCT3'
1b- 5'CTTCWTCGACTTYCAGACCCAAGGCAT3'	2f-5'GCATCCACCAWAWACYCTT3'
Ic-5'AAAGTGGGCAATACCCAACGC3'	2g-5'CCACTGTGTGCCCTTTGTTCCT3'
1d-5'TCACGCTTAGATGCTTTCAGCG3'	

Y=C+T, W=A+T

Primer sequences: *pfmdr1* genotyping

PCR step	**Primer sequence**
Primary PCR	MDR-A 5'GCGCGCGTTGAACAAAAAGAGTACCGCTG3'
	MDR-B 5'GGGCCCTCGTACCAATTCCTGAACTCAC3'
Secondary PCR	MDR-D1 TTTACCGTTTAAATGTTTACCTGC
	MDR-D2 CCATCTTGATAAAAAACACTTCTT

Primer sequences :*pfcrt* genotyping

PCR step	Primer sequence
Primary PCR	76-A 5' GCGCGCGCATGGCTCACGTTTAGGTGGAG3'
	76-B 5' GGGCCCGGCGGATGTTACAAAACTATAGTTACC3'
Secondary PCR	76-D1 5' TGTGCTCATGTGTTTAAACTT3'
	76-D2 5'CAAAACTATAGTTACCAATTTTG3'

Primer sequences :*dhfr* genotyping

PCR step	Primer sequence
Primary PCR	FR519A-5’GCGCGCTAATAACTACACATTTA3’
	FR519B5’-5’CCCGGGCTCTTATATTTCAATTT3’
Secondary PCR (codons 51 and 59)	FR51-D5’CTAGGAAATAAAGGAGTATTACCATGGAAATGGA3’
	FR59-D5’ATTTTTCATATTTTGATTCATTCACATATATGTTGTAACTGTAC3’
Secondary PCR (codon 108)	FR109-D5’CTAATTCTAAAAAATTACAAAATGT
	FR164-D35’TTTCTTTTCTAAAAATTCTTGATAAACAACGGAACCTCTTA

Primer sequences :sequencing of *pfmdr1* and *pfcrt*

Primer	**sequence**
Mdr1A *F*	5’ TGTATGTGCTGTATTATCAGGAGG3’
Mdr1A*R*	5’ AAGCCTCTTCTATAATGGACATGG3’
Mdr1BF	5’ GCATACTGTTATTAATTATGG3’
Mdr1BR	5’ CCTGTTTCTCCAACGATTGC3’
Mdr1CF	5’ GTTATATTTCAAGACCAAATGTACC3’
Mdr1CR	5’ CATCTTCCAATGTTGCATCTTC3’
CRT I F	5’AAACTTATTTTTAAAGAGATTAAGG3’
CRT I R	5’ ACTTACCTATATCTAATATTAAAAAGC3’
CRT II F	5’CAGAATTGTGGTCTTGGTATGG3’
CRT II R	5’ TTTATATCTTTTTAATTCTTACGG3’

Primers and probes :estimation of *pfmdr1* copy number

Primer/probe	**Sequence**
Pfmdr1-1F	5’-TGCATCTATAAA ACG ATC AGACAA A-3’
Pfmdr1-1R	5’-TCGTGTGTTCCATGTGACTGT-3’
B-tubulin-1F	5’-TGATGTGCGCAAGTGATCC-3’
B-tubulin-1R	5’-TCCTTTGTGGACATTCTTCCT C-3’
Pfmdr1 probe	*FAM*-5’-TTTAATAACCCTGATCGAAATGGAACCTTTG-3’-TAMRA
B-Tubulin probe	JOE-5’-TAGCACATGCCGTTAAATATCTTCCATGTCT-3’-TAMRA

Primer sequences :confirmation of microarray results by qPCR

Primer/probe	Sequence
PF10_0210F	5’ GCTGGAGGTGTGGGAGATT3’
PF10_0210R	5’ GGAACAAGCGATGAAGAACC3’
PF11_0172F	5’ AATCGAAGCCATAACGGAAA 3’
PF11_0172R	5’ CTCAATATGGTGGCAAAAACA3’
PFL1700cF	5’ TAGCTGACAATGCAGGAGGT 3’
PFL1700cR	5’ AAGTGCAGCCGAACCTACAC 3’
PFa0590wF	5’ TGGCTTATAAATATTTGGGCATC 3’
PFa0590wR	5’ TCCAAGGGTACTCAAAATTCG 3’
PFE1525wF	5’ CCAAGCGAATCAGACAAAAATAC 3’
PFE1525wR	5’ TGCTCATTTTTCTCATCCACA3’
PFE0825wF	5’ GCCAAATGACAAAACGAGTG3’
PFE0825wR	5’ AATCCCAATCCCAAGGCTAT3’
PF14_0331F	5’ GGCCAGAATTTTTAAGAAATGAA3’
PF14_0331R	5’ TACCAGCAACAAATCCACCA3’
PFE1150wF	5’ TGCCCACAGAATTGCATCTA3’
PFE1150wR	5’TTCATCGTGTGTTCCATGTG3’
PFB0105c F	5’TGGTGGAAATGTTGTGGTCA3’
PFB0105cR	5’ TGTTGTCCATGAATGCTTTATCA3’
PF10_0017F	5’ AAGAAATTCCACCAAAACGTG3’
PF10_0017R	5’ TCACAATCATATGGGTTATTC3’

Appendix 3

Table S1: Relationship between drug IC_{50} and mutation status at *pfmdr1-86* and *pfmdr1-1246*.*

Drug	WW (n=8)	WM (n=2)	MW (n=18)	MM (n=13)	P value
	MedianIC_{50}(nM) (IQR)	Median IC_{50}(nM) (IQR)	Median IC_{50}(nM) (IQR)	Median IC_{50}(nM) (IQR)	(K wallis)
CQ	30 (15-52)	40 (21-59)	38 (15-52)	52 (21-78)	0.3
PQ	19 (14-35)	84 (-)	30 (16-40)	24 (18-38)	0.1
LM	136 72-178	179 (-)	34 (25-68)	56 (29-84)	**0.002**
AQ	15 (10-21)	4 (-)	14 (10-21)	20 (16-24)	0.18
DEAQ	29 (18-38)	5 (-)	24 (20-40)	43 (31-52)	0.06
DHA	3 (2-4)	4 (-)	2 (2-2)	2 (2-3)	0.1

*WW, wild type *pfmdr1-86* and *pfmdr1-1246*; WM, wild type *pfmdr1-86* and mutant *pfmdr1-1246*;MW, mutant *pfmdr1-86* and wildtype *pfmdr1-1246*; MM, mutant *pfmdr1-86* and *pfmdr1-1246.*

Table S2: Relationship between drug IC_{50} and mutation status at *pfcrt-76* and *pfmdr1-86* with mixed genotypes contributing equally to mutant and wild type categories*.

Drug	WW	WM	MW	MM	P value
	MedianIC_{50}(nM) (IQR)	Median IC_{50}(nM) (IQR)	Median IC_{50}(nM) (IQR)	Median IC_{50}(nM) (IQR)	(K wallis)
CQ	11 (9-18)	15 (12-22)	52 (41-59)	73 (49-106)	0.0001
PQ	27 (21-35)	31 (21-49)	32 (18-59)	38 (20-56)	0.7198
LM	124 (93-202)	62 (42-85)	94 (67-133)	31 (25-49)	0.0001
AQ	7 (6-11)	14 (10-17)	12 (11-13)	18 (11-22)	0.0590
DEAQ	29 (24-35)	24 (18-33)	20 (20-24)	41 (29-52)	0.0330
DHA	2.3 (1.0-4.2)	1.8 (1.2-2.8)	2.3 (1.8-3.2)	2.0 (1.3-2.5)	0.5710

*WW, wild type *pfcrt-76* and *pfmdr1-86;* WM, wild type *pfcrt-76* and mutant *pfmdr-86*;MW, mutant *pfcrt-76* and wild type *pfmdr1-86*; MM, mutant *pfcrt-76* and *pfmdr1-86.*The numbers in each category were as follows: CQ WW 12, WM 35, MW 21, MM 48; PQ WW 11, WM 29, MW 15, MM 29; LM WW 11, WM 31, MW 16, MM 29; AQ WW 3, WM 7, MW 5, MM 26; DEAQ WW 3, WM 7, MW 5, MM 26; DHA WW 10, WM 31, MW 16, MM 28

Table S3: Relationship between drug IC_{50} and mutation status at *pfmdr1-1246* and *pfcrt-76.***

Drug	WW (n=11) MedianIC_{50}(nM) (IQR)	WM (n=11) Median IC_{50}(nM) (IQR)	MW (n=2) Median IC_{50}(nM) (IQR)	MM (n=12) Median IC_{50}(nM) (IQR)	P value (K wallis)
CQ	15 (11-20)	52 (44-56)	12 (11-14)	63 (48-79)	**0.0002**
PQ	37 (18-43)	18 (15-38)	21 (17-24)	27 (18-44)	0.12
LM	54 (38-146)	25 (25-68)	104 (101-108)	43 (27-66)	0.26
AQ	13 (10-18)	15 (10-24)	26 (15-37)	20 (16-22)	1.0
DEAQ	25 (21-38)	25 (21-40)	34 (33-36)	45 (35-52)	0.6
DHA	2 (1-5)	2 (2-2)	4 (2-5)	2 (2-3)	0.10

*WW, wild type *pfmdr1-1246* and *pfcrt-76*; WM, wild type *pfmdr1-1246* and mutant *pfcrt-76*;MW, mutant *pfmdr1-1246* and wildtype *pfcrt-76*; MM, mutant *pfmdr1-1246* and *pfcrt-76*

Table S4:*pfmdr1* alleles in Kilifi isolates and their frequencies.

pfmdr1 allele	Amino acid position					Frequency (%)
	86	***184***	***1034***	***1042***	***1246***	
I	N	Y	S	N	D	6 (9)
II	N	F	S	N	D	7 (11)
III	N	F	S	N	Y	1 (2)
IV	Y	F	S	N	D	3 (5)
V	Y	F	S	N	Y	1 (2)
VI	Y	Y	S	N	D	22 (34)
VII	Y	Y	S	N	Y	24 (38)

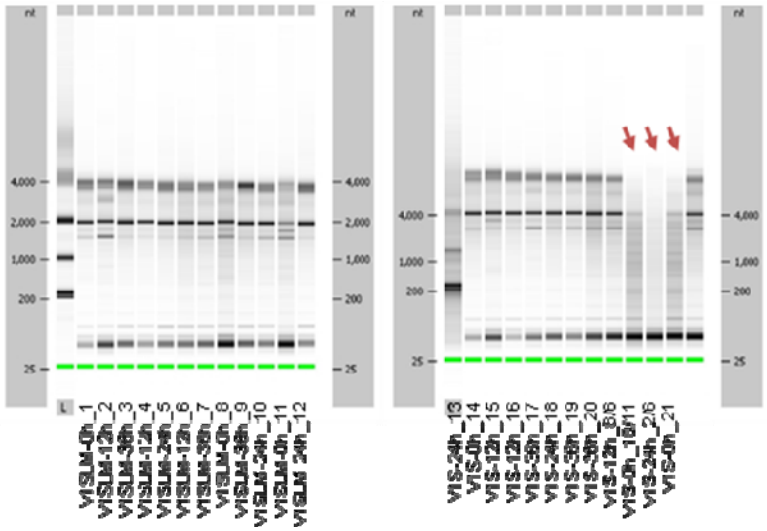
Appendix 4

Table S5: The frequencies of *pfcrt* and *pfmdr1* genotypes in isolates taken before and after treatment. Significant changes are shown in bold.

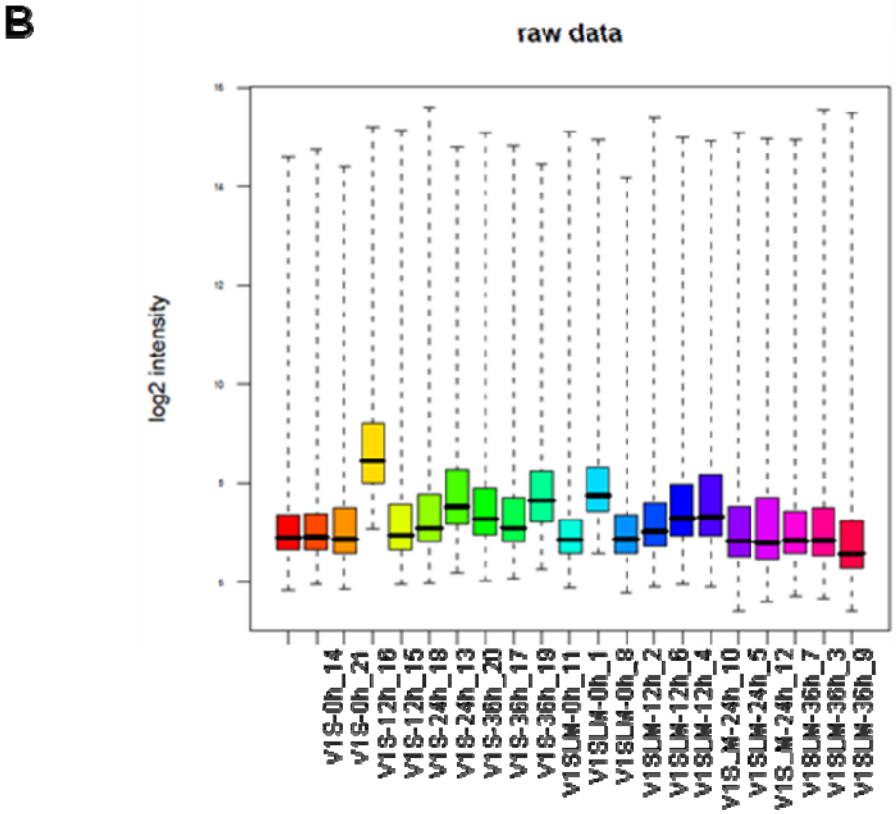
Treatment Group	Genotype		Frequency (Baseline)	Frequency (after treatment)	*P value (proportions test)*
DHA-PQ	*pfcrt-76*	K	7 (44)	9 (33)	0.44
		T	9 (56)	18(67)	0.47
	pfmdr1-86	N	5 (25)	9 (29)	0.74
		Y	15(75)	22(71)	0.75
	pfmdr1-184	F	3(21)	5(24)	0.84
		Y	11(79)	16(76)	0.84
	pfmdr1-1246	D	10(77)	7(50)	0.15
		Y	3(23)	7(50)	0.14
LM-ATM	***pfcrt-76***	K	1(10)	7(54)	0.08
		T	**9(90)**	**6(46)**	**0.03**
	pfmdr1-86	N	**1(9)**	**4(27)**	**0.0015**
		Y	10(91)	11(73)	0.24
	pfmdr1-184	F	0(0)	1(14)	0.30
		Y	7(100)	6 (86)	0.0044
	pfmdr1-1246	D	5(71)	3(50)	0.44
		Y	2(29)	3(50)	0.44
AQ	***pfcrt-76***	**K**	**11(35)**	**0(0)**	**0.004**
		T	**20(65)**	**18(100)**	**0.0002**
	pfmdr1-86	N	6(17)	4(24)	0.55
		Y	29(83)	13(76)	0.54
	pfmdr1-184	F	5(17)	0(0)	0.28
		Y	**25(83)**	**6(100)**	**0.0003**
	pfmdr1-1246	**D**	**22(69)**	**2(25)**	**0.02**
		Y	**10(31)**	**6(75)**	**0.02**

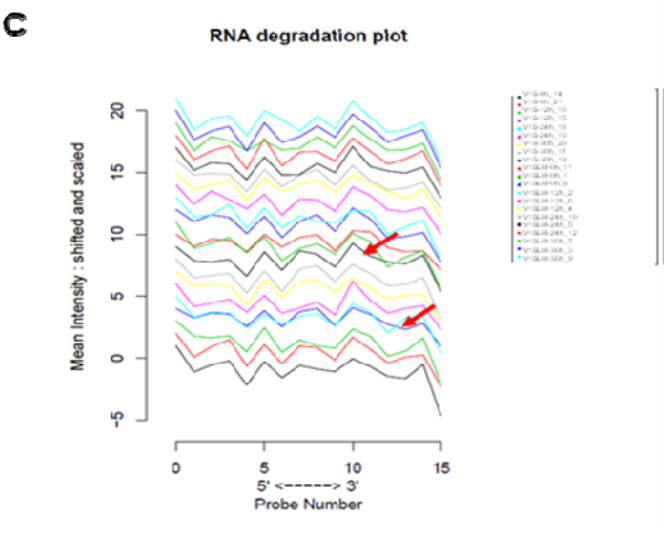
Appendix 5

A



B





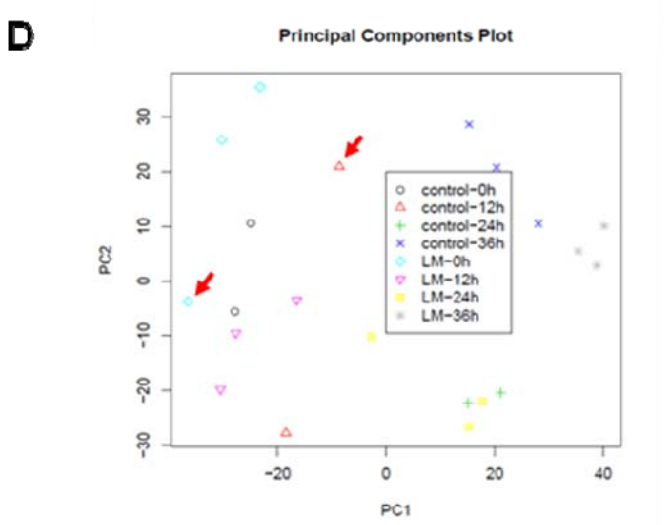


Figure S1: quality control of microarray samples. A) Agilent Bioanalyser electrophoresis file run summaries of total RNA showed that 3 of the 24 samples (red arrows) were degraded. Therefore these were not hybridised on PfSANGER arrays. B) Box plot analysis of the raw data post hybridization. C)RNA degradation plot and D)Prinicipal component analysis of the 21 samples prior to removal of outliers (indicated by arrows). Plots were generated in R using the 'affycoretools' package.

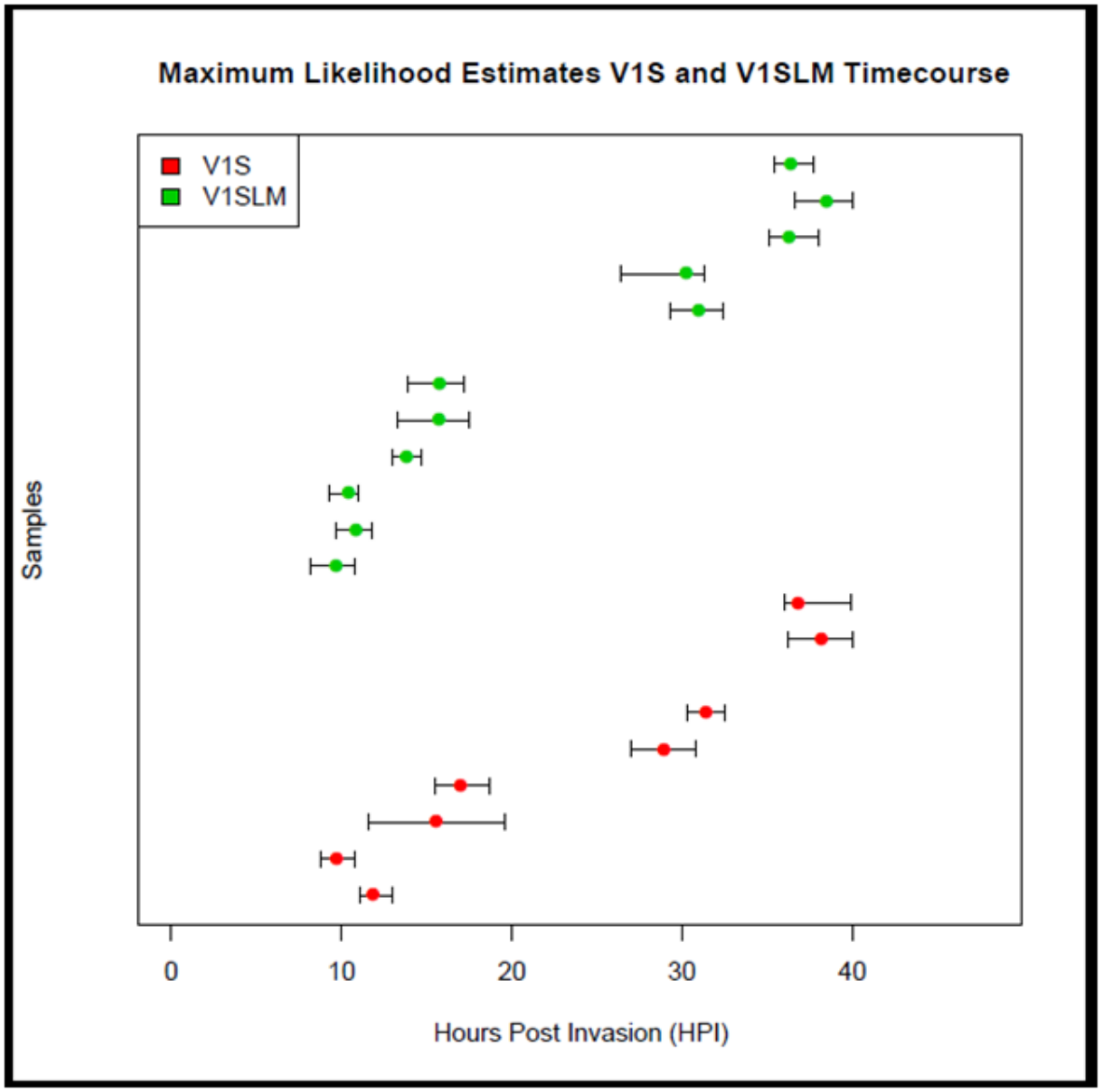


Figure S2: The intra-erythrocytic development cycle rates of V1S and V1S$_{LM}$. The maximum likelihood estimates (MLEs) and 95% confidence intervals of the samples are shown. Samples are ordered in the y dimension according to the MLE of hours post invasion (HPI). The MLEs of V1S and V1S$_{LM}$ samples are shown to be comparable across the time course.

Table S1: All genes with F adjusted P<0.05 (589 genes), an average expression AveExpr>log2(4) (371 genes), with a fold change of at least 1.5x (-0.58<log2Ratio<0.58; 266 genes) and with a Bayesian B>0 in at least one time point (184) are listed below. ID = *Plasmodium falciparum* gene ID. log2Ratio = log base 2 of fold change ratio between $V1S_{LM}$ and V1S. B = log odds of DE. AveExp = average expression of a given gene across the whole experiment. F.adj.P.Val = P values adjusted for multiple testing. Product description, TM (number of transmembrane domains), SP (presence of a signal peptide) and GO annotations were taken from www.plasmodb.org

	$V1S_{LM}$-V1S 0h		$V1S_{LM}$-V1S 12h		$V1S_{LM}$-V1S 24h		$V1S_{LM}$-V1S 36h									
ID	log2Ratio	B	log2Ratio	B	log2Ratio	B	log2Ratio	B	AveExpr	F. adj.P.Val	Product Description	TM	SP	GO Molecular Function	GO Biological Process	GO Cellular Component
MAL13P1.190	-0.97	0.39	-0.10	-5.90	-0.05	-5.88	0.42	-3.70	7.53	0.032	proteasome regulatory component, putative	0	null	enzyme regulator activity, endopeptidase activity	regulation of protein catabolic process, ubiquitin-dependent protein catabolic process	proteasome complex, proteasome regulatory particle
MAL13P1.21	0.12	-6.10	-0.24	-4.93	0.05	-5.84	0.63	1.55	4.62	0.028	conserved Plasmodium protein, unknown function	0	null	null	null	null
MAL13P1.235	-1.35	2.09	-0.12	-5.88	-0.18	-5.57	-0.22	-5.76	7.93	0.025	conserved Plasmodium membrane protein, unknown function	4	null	null	null	null
MAL13P1.257	-0.66	0.98	-0.22	-4.95	-0.06	-5.79	-0.19	-4.88	4.20	0.029	conserved Plasmodium protein, unknown function	0	null	null	null	null
MAL13P1.261	-0.75	1.05	-0.10	-5.82	-0.14	-5.35	0.09	-6.08	4.65	0.033	conserved Plasmodium protein, unknown function	0	null	null	null	null
MAL13P1.265	-0.71	0.35	-0.44	-3.31	-0.35	-3.29	-0.03	-6.39	5.17	0.025	conserved Plasmodium protein, unknown function	0	null	null	null	null
MAL13P1.271	-0.85	1.49	-0.28	-4.90	0.08	-5.77	0.32	-3.70	10.38	0.023	V-type ATPase, putative	4	yes	proton-transporting ATPase activity, rotational mechanism, ATP binding	ATP metabolic process, proton transport	vacuolar proton-transporting V-type ATPase complex, integral to membrane
MAL13P1.283	-0.63	1.90	-0.05	-5.92	0.12	-5.25	-0.32	-1.88	5.78	0.019	TCP-1/cpn60 chaperonin family, putative	0	null	ATPase activity, coupled, unfolded protein binding, ATP binding	protein folding	chaperonin-containing T-complex
MAL13P1.300	-0.75	1.73	-0.27	-4.63	-0.25	-4.09	0.08	-6.15	5.47	0.023	conserved Plasmodium protein, unknown function	3	yes	null	null	apicoplast
MAL13P1.345	-0.58	2.52	-0.14	-5.21	-0.13	-4.83	-0.15	-4.63	5.04	0.020	conserved Plasmodium protein, unknown function	0	null	null	null	null
MAL13P1.356	0.67	0.82	0.36	-3.74	0.12	-5.42	-0.24	-4.29	4.16	0.025	erythrocyte membrane protein 1, PfEMP1	0	null	receptor activity	pathogenesis	integral to membrane
MAL13P1.76	-0.61	2.76	-0.17	-4.92	0.09	-5.39	-0.17	-4.40	4.50	0.018	TFIIH basal transcription factor subunit	0	null	RNA polymerase II transcription factor activity, zinc ion binding, protein binding	DNA repair, regulation of transcription	nucleus
MAL13P1.79	-0.80	1.79	-0.02	-5.97	0.10	-5.65	0.27	-4.02	7.90	0.024	conserved Plasmodium protein, unknown function	0	null	null	null	null
MAL13P1.8	1.57	1.72	0.50	-4.90	0.06	-5.89	-0.94	-0.79	5.52	0.016	RIF pseudogene	1	null	null	null	null
MAL13P1.82	-0.87	0.93	-0.62	-2.32	-0.25	-4.70	0.29	-4.48	5.72	0.021	phosphatidylinositol synthase	3	yes	CDP-diacylglycerol-inositol 3-phosphatidyltransferase activity	phosphatidylinositol biosynthetic process	membrane
MAL13P1.93	-0.92	2.05	-0.02	-5.97	-0.06	-5.84	-0.20	-5.26	4.25	0.025	conserved Plasmodium protein, unknown function	0	null	null	null	null
MAL7P1.139	-0.90	3.81	-0.05	-5.91	0.00	-5.92	0.08	-6.10	5.15	0.017	mago nashi protein homolog, putative	0	null	null	sex determination	nucleus
MAL7P1.174	1.37	-3.00	3.11	1.85	2.66	2.41	1.57	-0.81	6.59	0.003	Plasmodium exported protein (PHISTb), unknown function	1	null	null	null	null
MAL7P1.175	0.68	-1.76	1.21	1.55	0.79	0.06	0.18	-5.75	4.23	0.011	Serine/Threonine protein kinase, FIKK family, pseudogene	0	null	protein serine/threonine kinase activity, ATP binding	protein amino acid phosphorylation	null
MAL7P1.300	-1.67	3.04	0.29	-5.53	-0.15	-5.71	-0.98	0.05	7.49	0.011	40S ribosomal protein S29, putative	0	null	structural constituent of ribosome	translation	ribosome
MAL7P1.56	0.65	0.06	0.24	-4.95	0.10	-5.61	-0.27	-4.02	4.01	0.032	erythrocyte membrane protein 1, PfEMP1	0	null	cell adhesion molecule binding, receptor activity	pathogenesis, antigenic variation, cell-cell adhesion, cytoadherence to microvasculature, mediated by parasite protein, rosetting	infected host cell surface knob, host cell plasma membrane, integral to membrane
MAL7P1.64	-0.70	3.26	-0.30	-3.48	-0.30	-2.38	-0.34	-1.26	4.13	0.008	serpentine receptor, putative, PfSR25	8	yes	null	null	null
MAL8P1.107	-1.00	2.29	-0.51	-3.18	-0.18	-5.23	-0.20	-5.42	4.98	0.019	conserved Plasmodium protein, unknown function	1	null	null	null	null
MAL8P1.142	-1.36	2.27	-0.15	-5.83	-0.22	-5.38	0.29	-5.26	9.65	0.023	proteasome beta-subunit	0	null	threonine-type endopeptidase activity, endopeptidase activity	ubiquitin-dependent protein catabolic process	proteasome core complex
MAL8P1.150	0.13	-6.26	0.07	-5.94	-0.23	-5.26	0.94	1.33	4.63	0.031	conserved Plasmodium protein, unknown function	0	null	phosphorus-oxygen lyase activity	intracellular signaling cascade, cyclic nucleotide biosynthetic process	null
MAL8P1.220	1.10	2.05	0.43	-4.27	0.18	-5.38	-0.42	-3.32	4.32	0.019	erythrocyte membrane protein 1, PfEMP1	0	null	receptor activity	pathogenesis	integral to membrane
MAL8P1.36	-1.14	6.70	-0.22	-4.92	-0.11	-5.49	-0.16	-5.14	5.71	0.004	conserved Plasmodium protein, unknown function	1	null	null	null	null
MAL8P1.82	0.16	-5.86	0.00	-5.98	0.04	-5.88	0.69	1.88	5.55	0.027	Vacuolar sorting protein VPS9, putative	0	null	null	null	null
MAL8P1.9	-0.76	1.99	0.10	-5.77	-0.06	-5.77	-0.44	-0.82	6.30	0.016	u6 snRNA-associated Sm-like protein, putative	0	null	U6 snRNA binding	nuclear mRNA splicing, via spliceosome	small nucleolar ribonucleoprotein complex
PF07_0013	-1.21	2.96	-0.22	-5.48	0.08	-5.80	-0.11	-6.15	4.50	0.021	conserved Plasmodium protein, unknown function	0	null	null	null	null
PF07_0048	1.11	2.06	0.42	-4.37	0.16	-5.49	-0.40	-3.67	4.24	0.020	erythrocyte membrane protein 1, PfEMP1	0	null	receptor activity, cell adhesion molecule binding	cell-cell adhesion, pathogenesis, cytoadherence to microvasculature, mediated by parasite protein, rosetting, antigenic variation	host cell plasma membrane, infected host cell surface knob, integral to membrane
PF07_0072	-0.14	-6.22	0.29	-5.25	-0.15	-5.61	0.91	1.49	5.26	0.029	calcium-dependent protein kinase 4	0	null	calcium-dependent protein serine/threonine phosphatase activity, protein tyrosine kinase activity, ATP binding, protein serine/threonine kinase activity, calcium ion binding	protein amino acid phosphorylation	null
PF07_0088	-1.06	1.68	0.09	-5.90	0.17	-5.46	-0.36	-4.09	7.63	0.024	40S ribosomal protein S5, putative	0	null	structural constituent of ribosome	translation	cytosolic small ribosomal subunit
PF08_0031	-1.49	0.01	-0.92	-3.50	0.11	-5.85	0.62	-4.04	6.58	0.028	oxoglutarate/malate translocator protein, putative	1	null	dicarboxylic acid transmembrane transporter activity, binding, oxoglutarate:malate antiporter activity	dicarboxylic acid transport, mitochondrial transport, malate transport, alpha-ketoglutarate transport	mitochondrial inner membrane
PF08_0053	-1.04	1.31	-0.30	-5.16	-0.05	-5.88	-0.33	-4.47	4.77	0.026	BRIX domain, putative	0	null	null	null	null
PF08_0058	0.38	-4.54	0.16	-5.71	-0.10	-5.76	0.94	2.83	4.67	0.020	MAC/Perforin, putative	0	null	null	null	null
PF08_0071	-1.16	0.08	-0.64	-3.90	-0.12	-5.78	0.13	-6.19	9.44	0.037	Fe-superoxide dismutase	0	null	superoxide dismutase activity, metal ion binding	response to oxidative stress, superoxide metabolic process	null
PF08_0082	0.23	-5.68	0.30	-5.05	-0.30	-4.52	1.06	3.82	4.22	0.013	conserved Plasmodium protein, unknown function	0	null	null	null	null

PF08_0110	-1.04	3.98	-0.08	-5.87	-0.25	-4.41	-0.15	-5.65	6.48	0.013	PfRab18, GTPase	0	null	protein binding, GTP binding, GTPase activity	nucleocytoplasmic transport, small GTPase mediated signal transduction, intracellular protein transport	intracellular
PF08_0124	-0.64	6.19	-0.13	-4.86	0.01	-5.91	-0.20	-2.17	5.06	0.004	conserved Plasmodium protein, unknown function	0	null	null	null	null
PF10_0004	1.46	2.86	0.45	-4.70	0.23	-5.37	-0.58	-2.67	4.52	0.015	rifin	1	null	molecular_function	antigenic variation	host cell plasma membrane, membrane
PF10_0005	0.71	2.00	0.22	-4.83	0.07	-5.74	-0.30	-2.96	4.24	0.020	rifin	1	null	molecular_function	antigenic variation	membrane, host cell plasma membrane
PF10_0015	2.46	4.46	1.22	-2.24	0.82	-3.05	1.64	2.50	4.55	0.002	acyl CoA binding protein, isoform 1, ACBP1	0	null	acyl-CoA binding	fatty acid metabolic process	cellular component
PF10_0016	1.95	-3.55	5.84	3.26	4.75	3.39	4.08	3.51	8.07	0.000	acyl CoA binding protein, isoform 2, ACBP2	0	null	acyl-CoA binding	fatty acid metabolic process	cellular component
PF10_0017	0.69	-3.21	0.83	-2.74	2.95	8.01	0.76	-1.39	4.29	0.000	Plasmodium exported protein (PHISTa), unknown function	1	null	molecular_function	biological_process	membrane
PF10_0019	2.47	-0.77	5.72	4.91	4.45	4.60	3.36	3.72	9.04	0.000	early transcribed membrane protein 10.1, etramp 10.1	2	yes	molecular_function	biological_process	cellular_component, membrane
PF10_0020	0.16	-6.20	0.32	-5.29	1.96	5.65	2.01	8.15	4.48	0.000	alpha/beta hydrolase, putative	0	null	null	null	null
PF10_0029	-0.35	-3.88	0.10	-5.81	0.71	1.41	0.00	-6.41	4.64	0.025	conserved Plasmodium protein, unknown function	0	null	thiol oxidase activity	oxidation reduction	null
PF10_0094	0.13	-6.37	0.09	-5.95	-0.27	-5.46	1.37	1.48	4.55	0.031	tubulin-tyrosine ligase, putative	0	null	tubulin-tyrosine ligase activity	protein modification process	null
PF10_0104	-0.69	1.06	-0.11	-5.73	0.01	-5.91	0.04	-6.36	4.72	0.036	conserved Plasmodium protein, unknown function	0	yes	null	null	membrane
PF10_0127	-0.58	1.75	-0.04	-5.93	-0.14	-4.87	-0.10	-5.68	4.50	0.025	conserved Plasmodium protein, unknown function	1	yes	protein binding, molecular_function, zinc ion binding	biological_process	membrane
PF10_0160	-0.87	1.53	-0.22	-5.28	0.07	-5.80	-0.28	-4.38	6.14	0.025	Serine/Threonine protein kinase, FIKK family	1	null	protein serine/threonine kinase activity, ATP binding, molecular_function	biological_process, protein amino acid phosphorylation	membrane, mitochondrion, cellular_component
PF10_0180	-0.75	1.14	-0.21	-5.24	-0.07	-5.76	-0.08	-6.20	7.31	0.032	conserved Plasmodium protein, unknown function	0	null	null	null	null
PF10_0181	-1.06	2.46	0.05	-5.95	-0.07	-5.83	-0.13	-5.97	4.19	0.024	conserved protein, unknown function	1	null	molecular_function	biological_process	membrane
PF10_0193	0.36	-4.85	0.17	-5.70	0.47	-3.14	1.26	5.19	5.02	0.007	microtubule-associated protein 1 light chain 3, putative	0	null	molecular_function	biological_process	cellular_component
PF10_0264	-0.91	0.01	-0.10	-5.88	-0.03	-5.90	-0.50	-2.70	7.86	0.031	40S ribosomal protein S2B, putative	0	null	structural constituent of ribosome	translation	cytosolic small ribosomal subunit
PF10_0266	-0.89	3.16	-0.37	-3.65	-0.14	-5.29	-0.05	-6.31	4.73	0.017	small subunit rRNA processing stabilizing factor, putative	0	null	null	null	null
PF10_0323	-1.45	0.31	-0.03	-5.98	-0.51	-4.44	0.04	-6.40	12.70	0.040	early transcribed membrane protein 10.2, etramp 10.2	2	yes	molecular_function	biological_process	membrane
PF10_0366	-0.99	0.98	-0.45	-4.21	-0.04	-5.89	-0.48	-2.74	9.73	0.023	ADP/ATP transporter on adenylate translocase	3	null	ATP:ADP antiporter activity, binding	transport	mitochondrial inner membrane
PF10_0401	1.16	1.59	0.37	-4.90	-0.12	-5.71	-0.06	-6.35	4.19	0.028	rifin	1	yes	molecular_function	antigenic variation	membrane, host cell plasma membrane
PF10_0406	0.96	0.36	0.51	-3.91	0.26	-5.00	-0.41	-3.77	4.07	0.025	erythrocyte membrane protein 1, PfEMP1	0	null	host cell surface receptor binding, receptor activity, cell adhesion molecule binding	antigenic variation, rosetting, cytoadherence to microvasculature, mediated by parasite protein, pathogenesis, cell-cell adhesion	integral to membrane, host cell plasma membrane, infected host cell surface knob
PF10_0415	-0.80	1.49	-0.13	-5.68	-0.22	-4.74	0.10	-6.06	4.73	0.027	conserved Plasmodium protein, unknown function	1	null	null	null	null
PF11_0021	1.57	1.53	0.69	-4.13	0.23	-5.55	-0.66	-3.20	4.32	0.021	rifin	2	yes	molecular_function	antigenic variation	host cell plasma membrane, membrane
PF11_0043	-0.60	0.30	-0.29	-4.26	-0.08	-5.68	-0.02	-6.40	4.47	0.037	60S ribosomal protein P1, putative	0	null	structural constituent of ribosome	translation, translational elongation	large ribosomal subunit, ribosome
PF11_0051	-0.66	2.06	-0.31	-3.65	0.01	-5.91	0.02	-6.38	6.26	0.023	phenylalanyl-tRNA synthetase beta chain, putative	0	null	ATP binding, RNA binding, phenylalanine-tRNA ligase activity	phenylalanyl-tRNA aminoacylation	phenylalanine-tRNA ligase complex, cytoplasm
PF11_0172	-0.63	-2.96	-0.16	-5.76	0.51	-3.19	0.84	0.44	7.51	0.023	folate/biopterin transporter, putative	11	null	molecular_function	biological_process	cellular_component
PF11_0309	-1.03	3.62	0.24	-5.09	-0.24	-4.53	-0.09	-6.11	4.72	0.015	conserved Plasmodium protein, unknown function	2	null	molecular_function	biological_process	membrane
PF11_0400	-0.66	0.27	-0.04	-5.94	-0.16	-5.20	-0.10	-6.03	5.35	0.044	conserved Plasmodium protein, unknown function	0	null	molecular_function	biological_process	membrane
PF11_0447	-0.66	0.44	-0.12	-5.68	-0.07	-5.76	0.05	-6.30	5.67	0.044	translation initiation factor eIF-1A, putative	0	null	translation initiation factor activity, RNA binding	translational initiation	eukaryotic 43S preinitiation complex
PF11_0458	-0.62	0.07	0.08	-5.85	-0.04	-5.87	-0.18	-5.14	4.10	0.044	conserved Plasmodium protein, unknown function	0	null	catalytic activity, RNA polymerase II transcription factor activity	transcription initiation from RNA polymerase II promoter	null
PF11_0464	0.14	-5.98	0.01	-5.98	-0.25	-4.43	0.59	0.51	4.37	0.036	Ser/Thr protein kinase, putative	0	null	ATP binding, protein kinase activity, protein tyrosine kinase activity, protein serine/threonine kinase activity	protein amino acid phosphorylation, biological_process	apicoplast, membrane
PF13_0006	1.14	1.37	0.56	-3.78	0.24	-5.17	-0.38	-4.30	4.32	0.023	rifin	1	yes	molecular_function	antigenic variation	host cell plasma membrane, membrane
PF13_0049	-0.68	1.16	0.19	-5.18	0.08	-5.66	0.09	-6.03	5.48	0.031	60S ribosomal protein L24, putative	0	null	structural constituent of ribosome	translation	cytosolic large ribosomal subunit
PF13_0051	-0.58	0.92	-0.20	-4.94	-0.13	-5.23	-0.22	-3.96	4.90	0.025	snornp protein gar1 homologue, putative	0	null	rRNA binding	rRNA processing, RNA modification	small nucleolar ribonucleoprotein complex
PF13_0128	-2.01	6.13	0.18	-5.74	-0.01	-5.91	-0.27	-5.37	5.43	0.006	beta-hydroxyacyl-ACP dehydratase precursor	1	yes	catalytic activity, (3R)-hydroxymyristoyl-[acyl-carrier-protein] dehydratase activity	fatty acid biosynthetic process	apicoplast
PF13_0214	-0.86	3.44	-0.19	-5.19	0.00	-5.91	-0.23	-4.34	7.47	0.016	elongation factor 1-gamma, putative	0	null	translation elongation factor	translational elongation	eukaryotic translation elongation factor 1
PF13_0282	-0.71	0.53	-0.47	-2.92	-0.27	-4.18	0.06	-6.30	4.62	0.025	proteasome subunit, putative	0	null	endopeptidase activity, threonine-type endopeptidase activity	ubiquitin-dependent protein catabolic process	proteasome core complex
PF13_0285	-0.07	-6.32	0.15	-5.55	0.07	-5.74	0.69	2.78	5.96	0.023	inositol-polyphosphate 5-phosphatase	3	null	inositol-polyphosphate 5-phosphatase activity	null	null
PF13_0300	-0.94	0.27	-0.43	-4.43	0.23	-5.15	0.00	-6.41	5.83	0.036	mitochondrial inner membrane translocase, putative	2	null	protein transporter activity	protein targeting to mitochondrion	mitochondrial inner membrane presequence translocase complex, mitochondrial inner membrane

PF13_0305	-1.31	4.99	-0.56	-2.78	-0.32	-4.01	-0.27	-4.68	5.76	0.006	elongation factor-1 alpha	0	null	GTP binding, GTPase activity, translation elongation factor activity	translational elongation	eukaryotic translation elongation factor 1 complex
PF13_0310	-0.61	0.59	-0.16	-5.37	-0.02	-5.90	0.07	-6.16	4.91	0.040	preribosomal processosome UTP, putative	0	null	null	null	null
PF13_0358	-1.92	1.99	-0.40	-5.47	0.23	-5.64	-0.22	-6.08	6.11	0.025	mitochondrial import inner membrane translocase, putative	0	null	P-P-bond-hydrolysis-driven protein transmembrane transporter activity	protein import into mitochondrial inner membrane, protein targeting to mitochondrion	mitochondrial intermembrane space protein transporter complex, mitochondrial intermembrane space
PF14_0003	0.97	0.98	0.42	-4.37	0.05	-5.87	-0.10	-6.18	4.31	0.031	rifin	3	yes	molecular_function	antigenic variation	membrane, host cell plasma membrane
PF14_0020	-1.42	1.96	-0.14	-5.86	0.04	-5.90	0.52	-3.59	7.68	0.023	choline kinase	0	null	choline kinase activity	lipid metabolic process	cellular_component
PF14_0071	-0.65	0.65	-0.11	-5.69	-0.15	-5.17	0.15	-5.41	4.48	0.033	conserved Plasmodium protein, unknown function	0	null	molecular_function	biological_process	apicoplast
PF14_0078	-0.78	2.50	-0.18	-5.31	0.01	-5.91	-0.38	-1.71	6.49	0.016	HAP protein	1	null	aspartic-type endopeptidase activity	proteolysis, biological_process	membrane
PF14_0143	-0.03	-6.44	0.07	-5.86	-0.04	-5.85	0.63	2.07	5.19	0.027	Atypical protein kinase, ABC-1 family, putative	0	null	null	null	null
PF14_0191	-0.85	1.66	-0.24	-5.12	-0.15	-5.36	0.14	-5.82	7.68	0.025	conserved Plasmodium protein, unknown function	0	null	null	null	null
PF14_0209	-0.64	0.36	-0.06	-5.90	-0.07	-5.75	0.08	-6.16	4.29	0.046	conserved Plasmodium protein, unknown function	0	null	null	null	null
PF14_0231	-1.00	0.27	-0.10	-5.90	-0.05	-5.88	-0.17	-5.91	5.69	0.047	60S ribosomal protein L7-3, putative	0	null	structural constituent of ribosome	ribosome biogenesis, translation	cytosolic large ribosomal subunit
PF14_0242	-1.11	1.56	-0.27	-5.35	-0.04	-5.90	-0.02	-6.41	5.94	0.030	arginine-N-methyltransferase, putative	0	null	arginine N-methyltransferase activity	biological_process, metabolic process	cellular_component
PF14_0293	0.12	-5.90	0.17	-5.22	-0.17	-4.79	0.65	3.78	4.07	0.015	conserved Plasmodium protein, unknown function	0	yes	null	null	null
PF14_0424	-1.06	1.82	-0.39	-4.50	0.07	-5.83	-0.10	-6.19	4.79	0.025	conserved Plasmodium protein, unknown function	1	null	molecular_function	biological_process	membrane
PF14_0425	-1.15	3.85	-0.32	-4.68	-0.09	-5.75	0.01	-6.41	11.86	0.015	fructose-bisphosphate aldolase	0	null	fructose-bisphosphate aldolase activity	gluconeogenesis, glycolysis	cellular_component
PF14_0429	-0.85	0.19	-0.02	-5.98	-0.04	-5.89	-0.17	-5.72	4.53	0.048	RNA helicase, putative	0	null	ATP-dependent helicase activity, nucleic acid binding, ATP binding, helicase activity	biological_process	cellular_component
PF14_0439	-0.93	0.91	-0.21	-5.50	0.31	-4.42	-0.34	-4.06	8.01	0.025	M17 leucyl aminopeptidase	0	null	manganese ion binding	proteolysis	apicoplast
PF14_0457	-0.41	3.33	-0.11	-4.86	-0.14	-3.35	-0.70	10.58	5.20	0.000	conserved Plasmodium protein, unknown function	0	null	null	null	null
PF14_0466	-0.58	0.97	-0.60	0.13	-0.30	-2.66	0.00	-6.41	4.53	0.013	Appr-1-p processing domain protein	0	null	null	null	null
PF14_0482	-0.80	0.64	-0.10	-5.84	-0.13	-5.55	-0.14	-5.83	6.01	0.038	conserved Plasmodium protein, unknown function	0	null	molecular_function	biological_process	cellular_component
PF14_0495	0.28	-5.70	0.10	-5.91	-0.21	-5.44	1.37	4.27	4.43	0.013	rhoptry neck protein 2	1	yes	molecular_function	biological_process	membrane
PF14_0586	0.08	-6.40	0.09	-5.94	-0.05	-5.89	1.14	1.54	4.59	0.031	conserved Plasmodium protein, unknown function	0	null	null	null	null
PF14_0619	-0.69	1.83	-0.25	-4.62	0.11	-5.48	-0.08	-6.06	4.35	0.025	conserved Plasmodium protein, unknown function	0	null	null	null	null
PF14_0627	-0.73	0.58	-0.17	-5.50	0.04	-5.87	-0.37	-2.84	6.38	0.026	40S ribosomal protein S3, putative	0	null	RNA binding, structural constituent of ribosome	translation	cytosolic small ribosomal subunit
PF14_0670	-0.31	-5.70	0.32	-5.36	-0.08	-5.86	-1.14	1.82	7.24	0.025	conserved Plasmodium protein, unknown function	0	null	null	null	null
PF14_0759	1.31	1.03	0.15	-5.85	0.25	-5.38	-0.82	-1.13	4.33	0.021	conserved Plasmodium protein, unknown function, pseudogene	1	yes	molecular_function	biological_process	membrane
PF14_0770	2.46	1.41	0.55	-5.45	0.37	-5.53	-1.49	-1.01	4.36	0.018	rifin	1	yes	molecular_function	antigenic variation	membrane, host cell plasma membrane
PFA0005w	0.86	0.44	0.20	-5.54	0.04	-5.88	-0.36	-3.81	4.00	0.031	erythrocyte membrane protein 1, PfEMP1	0	null	receptor activity, cell adhesion molecule binding	pathogenesis, cytoadherence to microvasculature, mediated by parasite protein, cell-cell adhesion, antigenic variation, rosetting	infected host cell surface knob, host cell plasma membrane, integral to membrane
PFA0010c	1.78	1.29	0.78	-4.21	0.20	-5.71	-0.65	-3.94	5.25	0.023	rifin	1	null	molecular_function	antigenic variation	membrane, host cell plasma membrane
PFA0050c	1.04	0.33	0.27	-5.41	0.20	-5.43	-0.53	-2.89	4.52	0.027	rifin	2	yes	molecular_function	antigenic variation	membrane, host cell plasma membrane
PFA0095c	0.98	1.76	0.26	-5.19	0.21	-5.13	-0.36	-3.68	4.27	0.022	rifin	1	yes	molecular_function	antigenic variation	host cell plasma membrane, membrane
PFA0230c	-0.66	-3.08	-0.01	-5.98	0.17	-5.60	-1.12	2.37	8.10	0.020	conserved Plasmodium protein, unknown function	0	null	null	null	null
PFA0590w	-0.08	-6.32	-0.33	-4.41	0.67	0.42	0.32	-3.60	5.28	0.027	ABC transporter, (CT family), putative, PfMRP1	11	null	ATPase activity, coupled to transmembrane movement of substances, ATP binding	transport	integral to membrane, membrane
PFA0615w	-0.63	-3.51	-0.20	-5.73	-0.55	-3.33	-4.25	14.26	4.68	0.000	Plasmodium exported protein, unknown function	2	yes	null	null	null
PFA0645c	2.32	1.54	0.22	-5.88	-0.07	-5.90	-1.23	-1.81	5.14	0.021	hypothetical protein	2	null	null	null	null
PFA0735w	-0.39	-5.36	-1.50	0.64	-1.18	0.60	-0.50	-4.06	4.32	0.013	Plasmodium exported protein (PHISTa), unknown function	1	yes	null	null	null
PFB0045c	1.50	2.08	0.56	-4.43	0.28	-5.25	-0.76	-1.75	4.04	0.016	erythrocyte membrane protein 1 (PfEMP1), truncated	2	null	null	null	null
PFB0085c	0.95	2.63	0.25	-5.05	0.04	-5.88	0.60	0.39	4.05	0.011	DNAJ protein, putative	1	null	unfolded protein binding, heat shock protein binding, molecular_function	protein folding, biological_process	host cell part
PFB0090c	1.30	-0.14	1.72	1.00	0.46	-4.55	0.35	-5.35	6.74	0.015	RESA-like protein with PHIST and DnaJ domains	1	yes	heat shock protein binding, unfolded protein binding, molecular_function	protein folding	cellular_component
PFB0100c	0.76	0.00	1.09	1.76	1.21	4.44	0.74	1.55	6.00	0.000	knob-associated histidine-rich protein	0	yes	null	null	host cell part
PFB0105c	0.82	-2.85	1.43	0.04	1.04	-0.58	1.37	2.61	5.25	0.005	Plasmodium exported protein (PHISTc), unknown function	1	null	molecular_function	biological_process	membrane
PFB0190c	-0.03	-6.43	-0.08	-5.86	-0.21	-4.67	0.58	0.86	4.11	0.033	conserved Plasmodium protein, unknown function	0	yes	transferase activity	null	null
PFB0245c	-0.73	0.17	0.11	-5.80	-0.13	-5.52	-0.23	-4.86	5.10	0.039	DNA-directed RNA polymerase II 16 kDa subunit, putative	0	null	nucleotide binding, DNA-directed RNA polymerase activity	transcription from RNA polymerase II promoter	cellular_component
PFB0290c	-1.79	0.76	-0.04	-5.98	-0.17	-5.78	-0.07	-6.39	6.16	0.042	transcription factor, putative	0	null	transcription factor activity, zinc ion binding	transcription from RNA polymerase III promoter, regulation of transcription	DNA-directed RNA polymerase III complex
PFB0445c	-0.70	3.13	0.20	-4.74	0.08	-5.62	-0.14	-5.16	6.10	0.018	DEAD box helicase, UAP56	0	null	nucleic acid binding, ATP binding, ATP-dependent RNA helicase activity	biological_process	cellular_component
PFB0595w	-0.84	0.10	-0.46	-3.92	-0.26	-4.79	-0.29	-4.61	5.82	0.028	heat shock 40 kDa protein, putative	0	null	heat shock protein binding, unfolded protein binding	response to unfolded protein, protein folding, response to heat	cellular_component

PFB0725c	-0.51	-4.22	-0.83	-2.30	-1.16	1.56	-0.40	-4.34	5.72	0.016	zinc finger protein, putative	4	yes	zinc ion binding	biological_process	apicoplast, membrane
PFB1045w	1.48	0.02	0.45	-5.28	0.40	-5.06	-0.08	-6.37	4.13	0.044	erythrocyte membrane protein 1 (PfEMP1), truncated	0	yes	null	null	null
PFC0005w	0.64	1.58	0.18	-5.12	0.07	-5.69	-0.26	-3.42	4.05	0.023	erythrocyte membrane protein 1, PfEMP1	0	null	receptor activity	pathogenesis	integral to membrane
PFC0030c	1.38	0.09	0.28	-5.66	0.25	-5.51	-0.61	-3.73	4.33	0.033	rifin	2	yes	molecular_function	antigenic variation	membrane, host cell plasma membrane
PFC0235w	-0.20	-4.55	0.10	-5.61	-0.10	-5.28	0.58	4.48	4.33	0.011	conserved Plasmodium protein, unknown function	0	null	actin binding, protein binding	cytoskeleton organization, vesicle-mediated transport	membrane
PFC0282w	-0.78	4.29	-0.02	-5.96	-0.10	-5.37	-0.34	-1.27	4.96	0.009	conserved Plasmodium protein, unknown function	2	yes	null	null	null
PFC0340w	0.01	-6.46	-0.82	0.72	-0.04	-5.87	0.05	-6.32	5.93	0.042	DNA polymerase epsilon subunit B, putative	0	null	DNA binding, DNA-directed DNA polymerase activity	DNA replication	delta DNA polymerase complex
PFC0395w	-0.72	1.53	0.14	-5.55	0.23	-4.32	-0.30	-3.17	6.76	0.021	asparagine synthetase, putative	0	null	asparagine synthase (glutamine-hydrolyzing) activity	asparagine biosynthetic process	null
PFC0725c	-0.91	1.14	-0.17	-5.63	0.04	-5.88	-0.02	-6.41	5.93	0.035	formate-nitrate transporter, putative	6	null	transporter activity	transport	membrane
PFC0840w	0.33	-4.92	-0.26	-5.24	0.13	-5.63	0.69	0.12	4.62	0.038	P-type ATPase, putative, PfATPase7	10	null	phospholipid-translocating ATPase activity, ATP binding, ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism, magnesium ion binding, transporter activity	phospholipid transport, cation transport	integral to plasma membrane
PFC0900w	-0.72	0.23	-0.38	-3.94	0.01	-5.91	-0.08	-6.20	6.76	0.037	T-complex protein 1 epsilon subunit, putative	0	null	ATP binding, unfolded protein binding	protein folding	chaperonin-containing T-complex
PFC1016w	-0.66	2.24	-0.06	-5.89	-0.07	-5.67	-0.06	-6.20	4.03	0.025	conserved Plasmodium protein, unknown function	0	null	null	null	null
PFC1120c	1.40	1.95	0.52	-4.46	0.20	-5.53	-0.58	-3.03	4.45	0.020	var (3D7-varT3-2)	0	null	receptor activity	pathogenesis	integral to membrane
PFD0090c	-0.27	-6.28	1.71	-2.09	2.33	1.64	0.97	-3.49	7.09	0.016	Plasmodium exported protein (PHISTa), unknown function	1	null	null	null	null
PFD0440w	-0.05	-6.35	-0.69	1.89	-0.15	-4.82	0.00	-6.41	4.17	0.025	peptidase, M22 family, putative	1	yes	endopeptidase activity, zinc ion binding	null	null
PFD0460c	-0.72	2.49	-0.20	-4.94	0.13	-5.21	-0.16	-5.15	4.43	0.021	conserved Plasmodium protein, unknown function	0	null	null	null	null
PFD0625c	0.85	0.55	0.28	-5.09	0.12	-5.63	-0.30	-4.34	4.04	0.030	erythrocyte membrane protein 1, PfEMP1	0	null	receptor activity, cell adhesion molecule binding	cytoadherence to microvasculature, mediated by parasite protein, pathogenesis, rosetting, cell-cell adhesion, antigenic variation	integral to membrane, infected host cell surface knob, host cell plasma membrane
PFD0835c	-0.86	8.49	0.01	-5.98	-0.06	-5.53	-0.22	-1.94	4.31	0.001	LETM1-like protein, putative	1	null	null	null	apicoplast
PFD1005c	1.04	0.81	0.54	-3.82	0.17	-5.52	-0.50	-2.98	4.08	0.023	erythrocyte membrane protein 1, PfEMP1	0	null	receptor activity, cell adhesion molecule binding	pathogenesis, cytoadherence to microvasculature, mediated by parasite protein, rosetting, cell-cell adhesion, antigenic variation	integral to membrane, host cell plasma membrane, infected host cell surface knob
PFD1140w	-2.06	3.50	-0.38	-5.43	-0.16	-5.76	-0.12	-6.28	7.75	0.018	Plasmodium exported protein (PHISTc), unknown function	1	yes	null	null	null
PFE0050w	-1.14	0.22	-0.20	-5.72	-0.06	-5.88	-0.28	-5.43	6.73	0.044	Plasmodium exported protein, unknown function	1	null	null	null	null
PFE0055c	-0.18	-6.19	0.15	-5.85	-0.06	-5.88	-0.96	0.36	4.83	0.047	heat shock protein, putative	1	null	heat shock protein binding, unfolded protein binding	protein folding	membrane, apicoplast
PFE0135w	-0.85	-4.40	-0.33	-5.72	-0.08	-5.89	-1.54	0.36	7.69	0.036	DNAJ domain protein, putative	0	null	heat shock protein binding	null	null
PFE0285c	-1.11	0.14	-0.29	-5.45	-0.15	-5.67	0.26	-5.50	8.68	0.042	small ubiquitin-related modifier, putative	0	null	null	protein ubiquitination during ubiquitin-dependent protein catabolic process, modification-dependent protein catabolic process	null
PFE0420c	0.16	-5.82	0.05	-5.92	-0.15	-5.22	0.64	1.86	4.34	0.025	guanidine nucleotide exchange factor	0	null	Ran guanyl-nucleotide exchange factor activity	DNA packaging	nucleus, apicoplast
PFE0440w	0.04	-6.43	-0.05	-5.95	-0.28	-4.50	0.67	0.43	4.63	0.039	conserved Plasmodium protein, unknown function	0	null	null	null	null
PFE0595w	-0.59	0.73	-0.23	-4.72	-0.01	-5.91	0.03	-6.35	4.60	0.036	prefoldin subunit, putative	0	null	unfolded protein binding	protein folding	prefoldin complex
PFE0630c	-1.67	2.08	-0.49	-4.98	0.14	-5.78	-0.24	-5.87	5.44	0.024	orotate phosphoribosyltransferase	0	null	orotate phosphoribosyltransferase activity	nucleoside metabolic process	null
PFE0790c	-0.89	0.28	-0.28	-5.22	0.09	-5.79	0.16	-5.84	9.41	0.042	BolA-like protein, putative	0	null	null	null	null
PFE0885w	-0.62	0.40	-0.13	-5.61	0.14	-5.29	-0.33	-2.61	6.34	0.025	eukaryotictranslation initiation factor 3 subunit, putative	0	null	RNA binding, translation initiation factor activity	translational initiation	null
PFE1035c	-0.98	4.04	-0.09	-5.82	-0.03	-5.89	-0.28	-3.67	4.09	0.013	BIS(5'-nucleosyl)-tetraphosphatase (Diadenosine tetraphosphatase), putative	0	null	bis(5'-nucleosyl)-tetraphosphatase (asymmetrical) activity	null	null
PFE1125w	1.00	1.35	0.31	-4.99	0.16	-5.50	-0.36	-3.97	4.76	0.024	mitochondrial ribosomal protein L17 precursor, putative	0	yes	structural constituent of ribosome	translation	mitochondrial large ribosomal subunit
PFE1240w	-0.96	0.56	-0.02	-5.98	-0.07	-5.84	-0.23	-5.39	5.00	0.039	conserved protein, unknown function	0	null	iron-sulfur cluster binding, iron ion binding, catalytic activity	null	null
PFE1340w	-0.87	0.95	-0.12	-5.80	-0.05	-5.87	-0.39	-3.12	5.62	0.025	conserved Plasmodium protein, unknown function	2	yes	null	transport	membrane; integral to membrane
PFE1370w	-0.64	0.48	-0.52	-1.85	-0.08	-5.69	-0.18	-5.08	5.85	0.023	hsp70 interacting protein, putative	0	null	null	null	null
PFF0030c	0.58	-1.21	0.88	0.87	0.40	-2.72	-0.42	-2.01	4.17	0.011	erythrocyte membrane protein 1(PfEMP1) pseudogene	0	yes	null	null	null
PFF0080c	2.56	4.69	0.83	-4.03	-0.43	-5.02	0.51	-4.84	4.02	0.010	TPR-like domain containing protein, putative	0	null	null	null	null
PFF1365c	0.19	-5.93	0.10	-5.87	-0.14	-5.59	1.11	4.00	4.21	0.015	HECT-domain (ubiquitin-transferase), putative	2	null	acid-amino acid ligase activity	protein modification process	intracellular
PFI0355c	-0.16	-5.93	0.10	-5.83	0.24	-4.66	0.74	2.01	4.27	0.024	ATP-dependent heat shock protein, putative	0	null	protein binding, ATP binding, ATPase activity	null	HslUV protease complex
PFI0620w	-1.85	0.27	-0.09	-5.96	0.55	-4.82	-0.08	-6.38	5.25	0.043	conserved Plasmodium protein, unknown function	0	null	null	null	null
PFI0720w	-0.01	-6.46	-0.41	-4.63	-0.19	-5.41	-0.77	0.09	11.26	0.042	transporter (MFS family), putative	11	null	null	null	null
PFI0785c	0.21	-4.34	0.17	-4.86	-0.01	-5.91	0.60	4.68	4.17	0.011	sugar transporter, putative	11	null	transporter activity	transport	integral to membrane
PFI0920c	0.28	-5.89	0.25	-5.65	0.17	-5.67	-1.01	0.09	6.58	0.047	dihydrouridine synthase, putative	2	yes	FAD binding, tRNA dihydrouridine synthase activity	tRNA processing	apicoplast
PFI1155w	0.34	-4.98	-0.30	-5.13	0.00	-5.92	0.82	1.11	5.37	0.029	conserved Plasmodium protein, unknown function	0	null	cyclin-dependent protein kinase regulator activity	cell cycle	null
PFI1175c	-0.79	0.21	-0.31	-4.85	-0.38	-3.47	-0.26	-4.76	6.78	0.026	RNA binding protein, putative	0	null	nucleic acid binding	null	null

PFI1370c	-0.77	1.51	0.03	-5.97	-0.13	-5.41	0.38	-2.22	8.24	0.022	phosphatidylserine decarboxylase	0	null	phosphatidylserine decarboxylase activity	phospholipid biosynthetic process	null
PFI1570c	-1.99	5.94	-0.55	-4.11	0.16	-5.63	0.16	-6.05	7.46	0.006	M18 aspartyl aminopeptidase	0	null	aminopeptidase activity, zinc ion binding	proteolysis	vacuole
PFI1675w	-0.89	0.60	0.26	-5.26	-0.12	-5.67	0.12	-6.09	4.32	0.038	conserved Plasmodium protein, unknown function	0	null	null	null	null
PFI1775w	0.91	4.86	0.10	-5.72	0.17	-4.76	-0.21	-4.32	4.01	0.010	lysophospholipase, putative	0	null	null	null	null
PFL0015c	1.26	0.56	0.36	-5.28	0.31	-5.09	-0.50	-3.97	4.49	0.028	rifin	1	yes	molecular_function	antigenic variation	membrane, host cell plasma membrane
PFL1645w	-0.58	1.26	-0.24	-4.42	-0.07	-5.66	0.16	-4.97	5.29	0.025	conserved Plasmodium protein, unknown function	0	null	null	null	null
PFL1665c	-0.61	3.62	0.00	-5.98	-0.07	-5.56	-0.08	-5.78	4.60	0.017	conserved Plasmodium protein, unknown function	0	null	null	null	null
PFL1700c	0.23	-5.94	0.27	-5.46	-0.16	-5.60	0.98	1.36	5.13	0.030	V-type K+-independent H+-translocating inorganic pyrophosphatase, PfVP2	16	null	hydrogen-translocating pyrophosphatase activity, inorganic diphosphatase activity	proton transport	membrane
PFL1870c	0.28	-5.83	0.25	-5.61	0.02	-5.91	1.20	2.12	4.90	0.025	sphingomyelin phosphodiesterase, putative	0	null	null	null	null
PFL1960w	0.84	0.42	0.36	-4.55	0.14	-5.52	-0.29	-4.46	4.10	0.030	erythrocyte membrane protein 1, PfEMP1	0	null	cell adhesion molecule binding, host cell surface receptor binding, receptor activity	pathogenesis, cell-cell adhesion, antigenic variation, rosetting, cytoadherence to microvasculature, mediated by parasite protein	host cell plasma membrane, integral to membrane, infected host cell surface knob
PFL2275c	-1.45	1.10	-0.66	-4.15	-0.01	-5.92	0.45	-4.60	7.72	0.025	FK506-binding protein (FKBP)-type peptidyl-propyl isomerase	0	null	FK506 binding, peptidyl-prolyl cis-trans isomerase activity	protein folding	cellular_component
PFL2370c	-0.73	-2.27	-1.15	0.04	-0.21	-5.36	0.57	-2.49	6.87	0.021	conserved Plasmodium protein, unknown function	0	null	null	null	null
PFL2505c	0.19	-5.93	0.22	-5.45	-0.10	-5.74	0.85	1.90	4.60	0.025	rhoptry neck protein 3, putative	3	yes	molecular_function	biological_process	membrane, apicoplast
PFL2665c	1.06	1.65	0.49	-3.88	0.10	-5.77	-0.43	-3.34	4.34	0.021	erythrocyte membrane protein 1, PfEMP1	0	null	receptor activity, host cell surface receptor binding, cell adhesion molecule binding	antigenic variation, cytoadherence to microvasculature, mediated by parasite protein, rosetting, pathogenesis, cell-cell adhesion	integral to membrane, infected host cell surface knob, host cell plasma membrane

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In Vitro Activities of Piperaquine, Lumefantrine, and Dihydroartemisinin in Kenyan *Plasmodium falciparum* Isolates and Polymorphisms in *pfcrt* and *pfmdr1*▽

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We have analyzed the in vitro chemosensitivity profiles of 115 Kenyan isolates for chloroquine (CQ), piperaquine, lumefantrine (LM), and dihydroartemisinin in association with polymorphisms in *pfcrt* at codon 76 and *pfmdr1* at codon 86, as well as with variations of the copy number of *pfmdr1*. The median drug concentrations that inhibit 50% of parasite growth (IC_{50}s) were 41 nM (interquartile range [IQR], 18 to 73 nM), 50 nM (IQR, 29 to 96 nM), 32 nM (IQR, 17 to 46 nM), and 2 nM (IQR, 1 to 3 nM) for CQ, LM, piperaquine, and dihydroartemisinin, respectively. The activity of CQ correlated inversely with that of LM ($r^2 = -0.26$; $P = 0.02$). Interestingly, parasites for which LM IC_{50}s were higher were wild type for *pfcrt-76* and *pfmdr1-86*. All isolates had one *pfmdr1* copy. Thus, the decrease in LM activity is associated with the selection of wild-type *pfcrt-76* and *pfmdr1-86* parasites, a feature that accounts for the inverse relationship between CQ and LM. Therefore, the use of LM-artemether is likely to lead to the selection of more CQ-susceptible parasites.

Chemotherapy is still the main approach for the control of malaria, and current strategies for malaria treatment rely on the use of combinations of drugs that include artemisinin compounds. Although this strategy is designed to reduce the chance of resistance emerging, there is considerable concern that this will inevitably occur.

For instance, the combination of lumefantrine (LM) and artemether (ATM), known as Coartem, has become the first-line treatment for malaria in many African countries, including Kenya (19). ATM is converted in vivo to dihydroartemisinin (DHA). Emerging reports indicate that the use of LM (in Coartem) selects for parasites that show increased tolerance to Coartem, and these parasites select for a wild-type *pfmdr1* genotype or show increased copy numbers of *pfmdr1*, a gene associated with chloroquine (CQ) and mefloquine (MFQ) resistance (7, 13, 15, 20, 36, 38). Thus, there is concern that resistance to LM could emerge rapidly. On the other hand, recent reports from Southeast Asia indicate that resistance to artemisinin derivatives is increasing, threatening the concept of artemisinin-based combinations (8).

Another combination, piperaquine (PQ) and DHA, known as Artekin, is undergoing clinical evaluation (17, 39, 42). This drug is efficacious, safe, and affordable and thus is likely to become an alternative to Coartem. PQ is a bisquinoline derivative consisting of two linked CQ molecules. Although reports indicate that PQ retains potency against CQ-resistant parasites (3), there is concern that PQ could become less susceptible against a backdrop of high CQ resistance (17, 22).

In this paper, we sought to analyze the in vitro activities of the antimalarials LM, DHA, and PQ in relation to polymorphisms in *pfcrt* at codon 76 (*pfcrt-76*) and in *pfmdr1* at codon 86 (*pfmdr1-86*) and in relation to *pfmdr1* copy number variations in Kenyan isolates. We used CQ as a reference drug.

MATERIALS AND METHODS

CQ was purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom). LM, PQ, and DHA were gifts from Steve Ward, Liverpool School of Tropical Medicine, Liverpool, United Kingdom.

Parasite adaptation. *Plasmodium falciparum* parasites were collected from malaria patients as part of several clinical studies that took place between 2005 and 2008 in the Kilifi district of Kenya. From the field, blood samples were collected, placed in "transport medium" (containing RPMI medium and albumin), and taken to the laboratory for long-term culture adaption, and other isolates were cryopreserved in liquid nitrogen before adaption. The detailed description of this long-term parasite adaptation procedure is given elsewhere (32). Provision of informed consent for the original studies was ensured by the Kenya Medical Research Institute, Nairobi.

Chemosensitivity testing. Routine cultures were carried out in RPMI 1640 medium (Gibco BRL, United Kingdom) supplemented with 15% (vol/vol) normal human serum, 25 mM bicarbonate, 2 mM glutamine, 25 mM HEPES buffer, and 3.6 nM *para*-aminobenzoic acid. The human blood used to culture the parasites was obtained from healthy subjects and was washed three times with RPMI culture medium that was not supplemented with serum. CQ was dissolved in distilled water, and LM and PQ were dissolved in 90% methanol plus 10% HCl; DHA was dissolved in 70% ethanol. The ranges tested (in a threefold dilution) were 7,180 to 0.12 nM for CQ and PQ, 17,505 to 0.3 nM for LM, and 163 to 0.22 nM for DHA. Assays were carried out in 200 μl of culture containing 0.5% parasitemia and 1.5% hematocrit, in 96-well microtiter plates. Cultures were incubated at 37°C in a gas mixture of 90% N_2, 5% CO_2, and 5% O_2 for 66 h. Thereafter, [^{3}H]hypoxanthine was added to the culture for another 18 h. Cells were then lysed by freeze-thawing; radiolabeled DNA was harvested on fiberglass paper (TomTec, Inc., and Perkin-Elmer); and the amount of ionizing radiation was determined using a Wallac 1450 MicroBeta counter.

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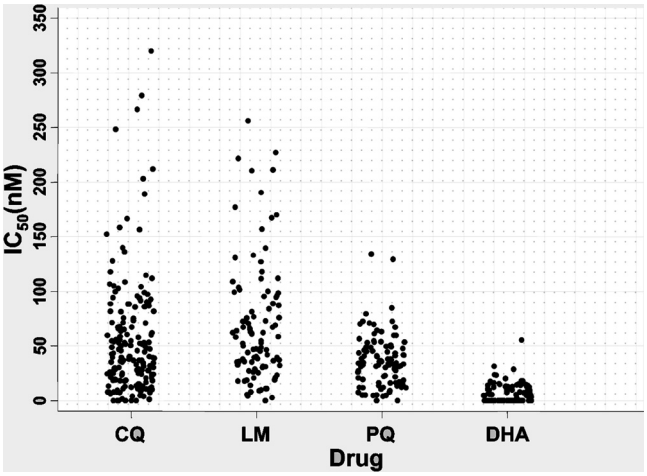

FIG. 1. In vitro activities of CQ, LM, PQ, and DHA against *Plasmodium falciparum* isolates.

Results were expressed as the drug concentration required for 50% inhibition of [^{3}H]hypoxanthine incorporation into parasite nucleic acid (IC_{50}), obtained by nonlinear regression analysis of the dose-response curve. We employed two reference *P. falciparum* laboratory strains: V1S, a multidrug-resistant strain, and 3D7, a drug-sensitive strain. IC_{50}s were measured two and four times for field isolates and reference strains, respectively.

Genotyping of *pfcrt* at codon 76 and of *pfmdr1* at codon 86. Blood samples (50 μl) of in vitro-adapted isolates were spotted onto filter paper, air dried, and stored in plastic bags with silica gel at ambient temperature. DNA was extracted by boiling, and single-base changes at *pfcrt-76* and *pfmdr1-86* were detected as reported previously (24).

Assessment of the copy number of *pfmdr1*. The *P. falciparum* reference isolates used in the analysis of *pfmdr1* copy numbers were DD2 (2 to 3 copies), D10 (1 copy), and 3D7 (1 copy). To estimate the copy numbers of *pfmdr1*, we used a relative-quantification TaqMan real-time PCR assay published elsewhere (29). In summary, the relative expression of *pfmdr1* in each isolate was compared with that of a housekeeping gene (β-tubulin) using specific fluorescently labeled probes (6-carboxyfluorescein and 2,7-dimethoxy-4,5-dichloro-6-caboxy-fluorescein) in a real time PCR assay. Every TaqMan run contained three calibrator DNA reference strains with known copy numbers. All assays were run in triplicate on an Applied Biosystems real-time PCR system, model 7500.

Statistical analysis. Statistical analyses were carried out using the Stata program, version 9 (Stata, Inc.). We compared differences between groups using the Kruskal-Wallis nonparametric test. We measured correlation using pairwise correlation analysis. All pairwise correlations of drug activity were estimated using log-transformed IC_{50}s. The level of significance was set at a P value of <0.05.

RESULTS

Chemosensitivity test. We first established the IC_{50}s of drugs against reference strains. Against V1S, the IC_{50}s of CQ, PQ, LM, and DHA were 158 ± 75, 42 ± 10, 24 ± 14, and 2 ± 1 nM, respectively; against 3D7, these values were 6.5 ± 2.3, 27 ± 17, 96 ± 12, and 2.0 ± 0.1 nM, respectively.

We have adapted 115 isolates for long-term culture and analyzed their drug chemosensitivity profiles. The median IC_{50}s of CQ, PQ, LM, and DHA were 41 nM (interquartile range [IQR], 18 to 73 nM), 32 nM (IQR, 17 to 46 nM), 50 nM (IQR, 29 to 96 nM), and 2 nM (IQR, 1 to 3 nM), respectively (Fig. 1). As expected, DHA was the most active drug, followed by PQ, CQ, and LM. Interestingly, LM IC_{50}s were >100 nM for about 20% of isolates (Fig. 1).

We have assessed the correlation between the drugs tested. LM activity correlated with DHA activity ($r^2 = 0.4$; $P < 0.0001$) and with PQ activity ($r^2 = 0.19$; $P = 0.05$). Interestingly, we observed an inverse relationship between the activities of CQ and LM ($r^2 = -0.26$; $P = 0.02$), suggesting that selection of LM resistance would be associated with an increase in CQ activity (Table 1).

TABLE 1. Correlation of in vitro antimalarial activities[a]

Drug 1	Drug 2	Correlation coefficient (r^2)	P
LM	**CO**	**−0.26**	**0.02**
LM	**PQ**	**0.19**	**0.05**
LM	**DHA**	**0.40**	**<0.0001**
PQ	CQ	0.16	0.13
PQ	DHA	0.04	0.69
DHA	CQ	0.14	0.16

[a] All pairwise correlations of drug activity were estimated using log-transformed IC_{50}s. Significant associations are shown in boldface.

***pfcrt-76*.** We also analyzed the *pfcrt-76* genotype in association with drug activity (Fig. 2). Based on CQ IC_{50}s, isolates can be classified into three distinct groups. For the first group, which has wild-type *pfcrt-76* ($n = 31$), the median IC_{50} of CQ is 13 nM (IQR, 10 to 15 nM). The second group is composed of mixed-genotype (wild-type and mutant) isolates ($n = 11$), for which the median CQ IC_{50} is 21 nM (IQR, 15 to 35 nM). The third group, which forms the majority of isolates ($n = 70$), consists of *pfcrt-76* mutants for which the median CQ IC_{50} is 57 nM (IQR, 46 to 80 nM). These differences are significant ($P < 0.001$) (Fig. 2). Based on the *pfcrt* genotype, more than 60% of isolates are resistant to CQ.

Median PQ IC_{50}s were very similar for *pfcrt-76* mutant (38 nM [IQR, 20 to 58 nM]) ($n = 39$) and wild-type (35 nM [IQR, 18 to 56 nM]) ($n = 23$) parasites. However, the median LM IC_{50} was lowest (43 nM [IQR, 27 to 90 nM]) for the *pfcrt-76* mutant group ($n = 40$), intermediate (56 nM [IQR, 42 to 84 nM]) for the mixed-genotype group ($n = 9$), and highest (67 nM [IQR, 48 to 111 nM]) for wild-type parasites ($n = 23$); these differences were statistically significant ($P = 0.03$) (Fig. 2). Thus, wild-type *pfcrt-76* parasites were associated with decreased susceptibility to LM, in line with the inverse relation-

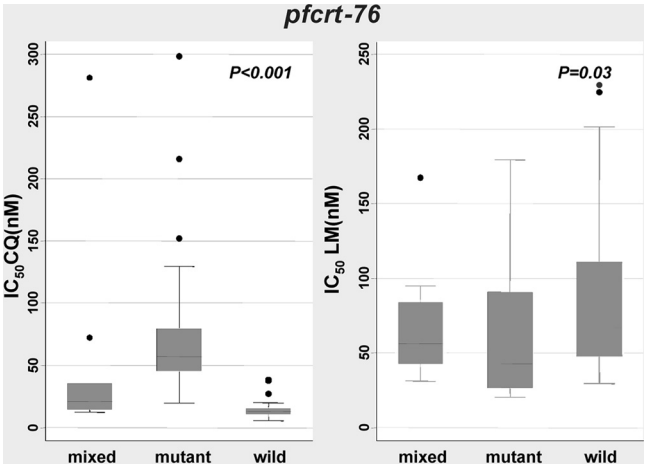

FIG. 2. Relationship between CQ and LM IC_{50}s and the *pfcrt* genotype at codon 76.

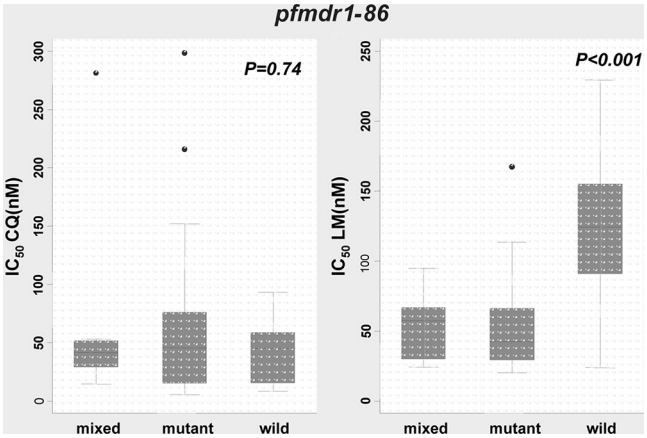

FIG. 3. Relationship between CQ and LM IC_{50}s and the *pfmdr1* genotype at codon 86.

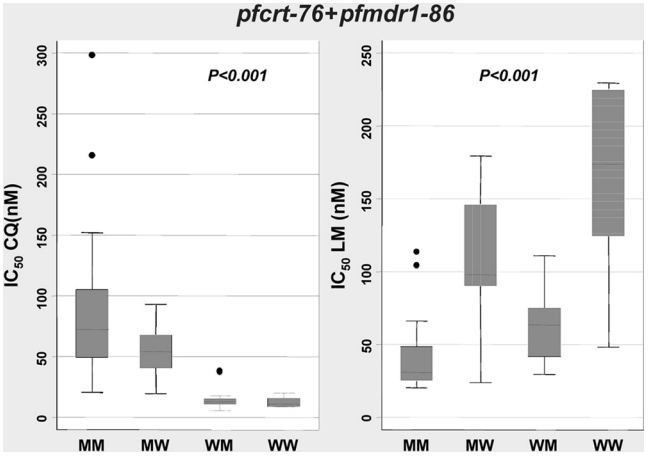

FIG. 4. Relationship between CQ and LM IC_{50}s and the *pfcrt-76* and *pfmdr1-86* genotypes at codons 76 and 86, respectively. MM, mutant *pfcrt-76* and *pfmdr1-86*; MW, mutant *pfcrt-76* and wild-type *pfmdr1-86*; WM, wild-type *pfcrt-76* and mutant *pfmdr1-86*; WW, wild-type *pfcrt-76* and *pfmdr1-86*.

ship with CQ resistance. DHA IC_{50}s did not differ as a function of the *pfcrt-76* genotype.

***pfmdr1-86*.** Median CQ IC_{50}s were similar for wild-type and mutant *pfmdr1-86* parasites (Fig. 3). However, the median DHA IC_{50} was higher (though marginally so) for *pfmdr1* wild-type isolates ($n = 23$) than for mutant isolates ($n = 47$) (3 nM [IQR, 3 to 4 nM] versus 2 nM [IQR, 1 to 3 nM]; $P = 0.04$). Interestingly, as in the case of *pfcrt-76*, the median LM IC_{50} was highest (124 nM [IQR, 90 to 155 nM]) for *pfmdr1-86* wild-type parasites ($n = 17$), intermediate (57 nM [IQR, 30 to 67 nM]) for mixed genotypes ($n = 8$), and lowest (43 nM [IQR, 29 to 66 nM]) for mutant isolates ($n = 47$); these differences were statistically significant ($P < 0.001$) (Fig. 3). Thus, *pfmdr1-86* contributes significantly to reduced susceptibility to LM.

***pfcrt-76* and *pfmdr1-86*.** We also investigated the effects of both *pfcrt-76* and *pfmdr1-86* mutations in relation to drug activity. To do so, we excluded parasites with mixed genotypes (at *pfcrt-76* or *pfmdr1-86*). Parasites were classified as *pfcrt-76* mutant and *pfmdr1-86* mutant (M-M), *pfcrt-76* mutant and *pfmdr1-86* wild type (M-W), *pfcrt-76* wild type and *pfmdr1-86* mutant (W-M), or wild type at both genes (W-W). The median CQ IC_{50} for parasites with the M-M genotype ($n = 45$) was significantly higher (72 nM [IQR, 49 to 105 nM]) than those for all others; the next highest median CQ IC_{50} (54 nM [IQR, 41 to 68 nM]) was that for M-W parasites ($n = 15$), and, as expected, the lowest CQ IC_{50}s (13 nM [IQR, 11 to 15 nM] and 11 nM [IQR, 9 to 15 nM]) were observed for W-M ($n = 21$) and W-W ($n = 6$) parasites, respectively (Fig. 4). Likewise, the median LM IC_{50} for M-M parasites ($n = 25$) was significantly lower (31 nM [IQR, 25 to 48 nM]) than those for M-W (99 nM [IQR, 90 to 146 nM]) ($n = 11$) and W-M (63 nM [IQR, 41 to 75 nM]) ($n = 17$) parasites. The highest median LM IC_{50}, 173 nM (IQR, 124 to 224 nM), was observed for W-W parasites ($n = 6$). These observations were statistically significant ($P < 0.001$) (Fig. 4). Thus, as expected, the presence of mutant genotypes in *pfcrt76* and *pfmdr186* is strongly associated with CQ resistance but also with increased susceptibility to LM.

Analysis of *pfmdr1* copy number. To gain more insight into the mechanism of decreased susceptibility to LM, we analyzed *pfmdr1* copy number variations in association with drug chemosensitivity profiles. We used the reference strain Dd2, which has 3 *pfmdr1* copies, though 2 copies have also been reported (5, 41), and the single-copy-number strains D10 and 3D7. Since we did not know the exact copy number for our Dd2 clone, we classified our data as isolates with 1 copy versus those with more than 1 copy. We eliminated data where threshold cycle (C_T) values were >35 and $\Delta\Delta C_T$ values were >1.5.

All 78 isolates analyzed for *pfmdr1* amplification were estimated to have one single copy of *pfmdr1*. Thus, no association could be established between *pfmdr1* amplification and drug activities.

DISCUSSION

Coartem has been adopted in many parts of Africa, including Kenya, as the first-line treatment against malaria. We have generated baseline information on the in vitro susceptibilities of *P. falciparum* isolates in Kilifi, an area of endemicity on the Kenyan coast, to LM and DHA. LM IC_{50}s are around 50 nM for most of the isolates; however, it is noteworthy that IC_{50}s for more than 20% of the isolates are >100 nM. The in vitro activity of LM against field isolates from several areas where malaria is endemic has been investigated using the WHO microtest. In all these studies, LM activity was high, with IC_{50}s of <30 nM for more than 95% of isolates (1, 4, 16, 21, 25, 26). In our study, we observed a higher LM IC_{50} range, and IC_{50}s of >200 nM for some isolates, findings similar to those of a report from Senegal (27).

Previous reports have indicated that the use of Coartem is associated with the selection of parasites with wild-type *pfmdr1-86*, which are tolerant of low LM concentrations (7, 15, 36–38). Our data clearly indicate that the presence of wild-type *pfmdr1-86* is associated with an increase in the LM IC_{50} (reduced susceptibility) and that this increase is more pronounced for parasites harboring a wild-type genotype at *pfcrt-76*, in line with a recent report (37). Since this genotype is associated with CQ susceptibility, it is not surprising that an inverse relationship

exists between CQ and LM activity, a feature that has been reported in other areas where malaria is endemic (27, 30).

We also investigated variation in the copy number of *pfmdr1*. There was no evidence of *pfmdr1* amplification in our isolates. This is consistent with previous data showing that *pfmdr1* amplification is a relatively rare event in African isolates compared with those in Southeast Asia (14, 36, 37, 40, 41); the high rate of parasites with more than 1 *pfmdr1* copy in Southeast Asia is likely due to the use of MFQ (23, 28, 29, 33). In Africa, MFQ is not used routinely for malaria treatment. Thus, under these conditions, one would expect parasites with multiple *pfmdr1* copies to be rare in Africa. In Southeast Asia, reduced LM susceptibility has been associated with an increase in the *pfmdr1* copy number (30); thus, it is possible that the continuous use of Coartem would select for parasites with increased *pfmdr1* copy numbers.

The significant decrease in LM susceptibility in vitro for parasites with wild-type *pfmdr1* and *pfcrt* genotypes that we report here is in line with previous work showing the emergence of LM-tolerant parasites after a single use of Coartem (7, 13, 15, 20, 36–38). However, it should be borne in mind that Coartem is effective at treating malaria, with high success rates (>90 to 95%), in many areas where malaria is endemic, including Kenya (11, 12, 18, 42). Thus, the mechanisms of Coartem resistance in vivo will likely involve other genes. Nevertheless, we propose that an increase in the frequency of parasites that are wild type for both *pfcrt-76* and *pfmdr1-86* (and probably in the *pfmdr1* copy number) in the population would be the first step in the selection of resistance to LM and that these parasites will form the backdrop for LM resistance and thus for Coartem resistance.

Artekin is now undergoing clinical evaluation and is likely to become an alternative to Coartem (17, 39, 42). PQ is active against Kilifi isolates, with IC_{50}s falling between 2 and 132 nM, and IC_{50}s of >100 nM were found for only two of the isolates tested. Similar results were obtained for isolates from other areas of malaria endemicity (2, 3, 6). We also demonstrate that polymorphism in *pfcrt-76* and *pfmdr1-86* is not associated with decreased susceptibility to PQ. However, a recent study using transgenic parasite lines has indicated that the presence of the *pfcrt* mutant decreases susceptibility to PQ in vitro (22), and in vivo, Artekin efficacy may be reduced in an area of high CQ resistance in Southeast Asia (17). In Africa, however, this drug remains effective, probably due to the lower level of CQ resistance in Africa than in Southeast Asia (17, 39, 42).

We have also generated data on the activity of DHA. As expected, this drug is very active against *P. falciparum*; most IC_{50}s are <25 nM. Our data indicate that wild-type *pfmdr1-86* parasites are less susceptible to DHA than mutant parasites (although this difference is marginal), in line with previous reports (9, 10, 31, 34). Likewise, previous investigations have indicated that an increase in the *pfmdr1* copy number and the presence of wild-type *pfcrt* genotypes are associated with reduced artemisinin susceptibility (20, 29, 33, 35). If this feature is translated in vivo, the emergence of LM resistance could be associated with a decrease in DHA efficacy. However, it remains to be seen whether the same would happen in vivo.

In conclusion, using laboratory-adapted African isolates, we have provided data showing that parasites that are less susceptible to LM in vitro are predominantly *pfmdr1* and *pfcrt* wild type. We propose that such parasites would form a backdrop on which LM resistance may thrive. Since Coartem has been rolled out in Africa, it is therefore critical to monitor LM and DHA activity in vitro and polymorphism in *pfcrt* and *pfmdr1*.

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Chloroquine resistance before and after its withdrawal in Kenya

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Abstract

Background: The spread of resistance to chloroquine (CQ) led to its withdrawal from use in most countries in sub-Saharan Africa in the 1990s. In Malawi, this withdrawal was followed by a rapid reduction in the frequency of resistance to the point where the drug is now considered to be effective once again, just nine years after its withdrawal. In this report, the polymorphisms of markers associated with CQ-resistance against *Plasmodium falciparum* isolates from coastal Kenya (Kilifi) were investigated, from 1993, prior to the withdrawal of CQ, to 2006, seven years after its withdrawal. Changes to those that occurred in the dihydrofolate reductase gene (*dhfr*) that confers resistance to the replacement drug, pyrimethamine/sulphadoxine were also compared.

Methods: Mutations associated with CQ resistance, at codons 76 of *pfcrt*, at 86 of *pfmdr1*, and at codons 51, 59 and 164 of *dhfr* were analysed using PCR-restriction enzyme methods. In total, 406, 240 and 323 isolates were genotyped for *pfcrt*-76, *pfmdr1*-86 and *dhfr*, respectively.

Results: From 1993 to 2006, the frequency of the *pfcrt*-76 mutant significantly decreased from around 95% to 60%, while the frequency of *pfmdr1*-86 did not decline, remaining around 75%. Though the frequency of *dhfr* mutants was already high (around 80%) at the start of the study, this frequency increased to above 95% during the study period. Mutation at codon 164 of *dhfr* was analysed in 2006 samples, and none of them had this mutation.

Conclusion: In accord with the study in Malawi, a reduction in resistance to CQ following official withdrawal in 1999 was found, but unlike Malawi, the decline of resistance to CQ in Kilifi was much slower. It is estimated that, at current rates of decline, it will take 13 more years for the clinical efficacy of CQ to be restored in Kilifi. In addition, CQ resistance was declining before the drug's official withdrawal, suggesting that, prior to the official ban, the use of CQ had decreased, probably due to its poor clinical effectiveness.

Background

From the 1940s up to the 1990s, chloroquine (CQ) was the mainstay of malaria therapy worldwide. The selection of *Plasmodium falciparum*-resistant isolates was first reported in South-East Asia and South America in the 1950s [1] and by the 1970s, CQ was no longer effective in these parts of the world. In Africa, CQ-resistant isolates only emerged in the 1970s. However, within 10 years, the level of resistance to CQ had risen rapidly [1], both in southern and eastern Africa [2]. In 1993, Malawi was the first African country to replace CQ as the first-line treatment of uncomplicated malaria with the antifolate combination sulphadoxine/pyrimethamine (SP) [3,4]. In 1999, Kenya also replaced CQ with SP [5]. Other countries, such as Uganda and Tanzania followed suit soon after [6,7].

Recent studies in Malawi have indicated that CQ resistance may revert to sensitivity within a decade of withdrawal of the drug, based on analysis of the population frequencies of the mutations known to cause or be associated with CQ resistance, *pfcrt* and *pfmdr1* genes [3], and assessment of *in vitro* activity and *in vivo* efficacy of the drug [8,9]. This reversal indicates that the *pfcrt* mutants may be loaded with a substantial fitness cost in the absence of drugs, thus leading to their decline in frequency once drug pressure is removed. This raises the possibility of re-introducing this safe and affordable drug for treatment of malaria in Africa [4]. In this paper, are reported the analyses of markers associated with CQ resistance (*pfcrt* and *pfmdr1*) and the *in vitro* activity of CQ against *P. falciparum* isolates collected in an area of high transmission in coastal Kenya (Kilifi), from 1993, prior to the removal of CQ, to 2006, seven years after its withdrawal. In the same area, these changes that occurred in the dihydrofolate reductase gene (*dhfr*), which confers resistance to the replacement drug SP were also compared [10,11].

Methods

Samples

A retrospective analasysis was carried out on blood samples collected from patients during several clinical trials of anti-malarial drugs conducted in Kilifi from 1993 to 2006. The 1993–2003 samples originated from several clinical trials of SP, chlorproguanil/dapsone (Lapdap™), and SP-artesunate [12-15] and were collected before drug treatment. Others were collected in 2006 from infected patients both before and after treatment in clinical trials of artemether/lumefantrine (AL)(Coartem™) versus piperaquine-dihydroartemisinin (Artekin®) [unpublished data], and amodiaquine (AQ) [16]. No samples were collected in 1996, 2004 and 2005. Blood samples (~50 μl) were spotted onto filter paper, air-dried and stored in plastic bags with silica gel at ambient temperature. Around thirty samples were selected per year and analysed for *P. falciparum* mutation at codon 76 (Lys to Thr) of *pfcrt (pfcrt-76)* and at codon 86 (Asn to Tyr) *of pfmdr1 (pfmdr1–86)*, and at codons 51,59 and 164 of *dhfr*.

Parasite adaptation and drug assays

A subset of the 2006 samples were adapted *in vitro* for long-term culture and their chemosensitivity profiles to CQ were analysed. Briefly, parasite adaptation was carried out as follows: 1 ml of patient venous blood was collected and added to 5 ml of transport medium (RPMI-1640 containing 10% albumin). Parasites in this medium could be kept at 4°C for 4 days before the *in vitro* adaptation or cryopreserved in liquid nitrogen and adapted later. Fresh parasites were put in culture after washing the suspension with normal RPMI-1640 (centrifugation at 2500 *g* for 15 min); cryopreserved parasites were thawed, washed and put into *in vitro* culture according to standard protocols of cryopreservation [16]. Parasitaemias were monitored until a per cycle increase in parasitaemia greater than two-fold was reached, at which point the culture was diluted by a factor >2 and if parasitaemia increased again (greater than two-fold), the culture was then considered "adapted": this process lasted two to eight weeks. Once parasites were adapted, their chemosensitivity to CQ was determined by the radioisotope incorporation method [17]. Results were expressed as the drug concentration required for 50% inhibition of [^{3}H] hypoxanthine incorporation into parasite nucleic acid (IC_{50}), using regression analysis of the dose-response curve. Fifty microlitres of culture from these adapted parasites was spotted onto filter paper for DNA extraction and genotyping.

Genotyping

Parasite genomic material from filter paper was prepared using the methanol procedure described elsewhere [18]. The detection of single-base changes at *pfcrt-76* and *pfmdr1–86* was carried out using the PCR-restriction enzyme protocol described elsewhere [3]. Point mutations in codons 51, 59 and 164 of *dhfr* were analysed as described elsewhere [18,19]. The analysis of mutations at codon 164 of *dhfr* of samples was not carried out routinely, since previously reports showed that this mutation was not present at detectable levels in our study site and other African sites where SP resistance is high [20,21]. However, in light of reports on the emergence of 164 *dhfr* mutant in Africa [22-24], the 164 codon of *dhfr* in isolates collected in 2006 was analysed. Mutation at codon 108 was not analysed since the presence of a mutation at codon 51 and/or 59 indicates that the parasite carries the resistant mutation at codon 108 [10,11].

Statistical analyses

Frequencies of mutations at *pfcrt-76*, *pfmdr1–86* and *dhfr* (codons 51 and 59) alleles were calculated as the propor-

tion of samples carrying the mutant form out of the total of all samples carrying either only the mutant form or only the wild-type form: thus samples carrying both wild-type and mutant forms, in which relative frequencies could not be determined, were excluded from the denominator and numerator. In the case of *dhfr*, those samples with either a mutation at *dhfr-51* (from the amino acid Asn to Ile) or *dhfr-59* (from Cys to Arg) were denoted as 'double mutants'. Those with mutations at both *dhfr-51* and *dhfr-59* were denoted as 'triple mutants' and those with or without a resistant allele mutation at *dhfr*-108 (Ser to Asn) were pooled and denoted as wild-type/single mutants ('WT/single mutants'). The data were first analysed by treating these as three haplotypes, and then by treating the double mutant forms as two separate alleles to give four haplotypes.

As some of the samples were collected from patients enrolled in clinical trials, an analysis was performed to determine the potential bias to population frequencies caused by drug treatment of the patients from which the parasites were sampled. This was done by comparing the frequencies of each mutant before versus after drug treatment using a Fisher's Exact test for each trial separately. Thereafter, an analysis was carried out for trends in the frequencies of mutants through time with and then without the post-treatment samples included in the data, by fitting a logistic regression model to the frequency data with generation as a linear covariate and assuming three parasite generations per year (an average of previously used estimates [25,26] and consistent with estimates of durations of infection in children [27]). The selection coefficient, *s*, which describes how much more (or less) fit the resistant mutant is relative to the wild-type, and is a function of the amount of drug pressure and also the cost of resistance in the absence of drug pressure [28], was calculated from the estimated slope of the logistic regression curve fitted to the observed allele frequencies over the generations, viz:

$$\log\left(\frac{p_R^n}{p_S^n}\right) = \log\left(\frac{p_R^0}{p_S^0}\right) + n\log\left(\frac{w_R}{w_S}\right) \qquad (1)$$

where the slope of this line is $\log\left(\frac{w_R}{w_S}\right) = 1 + s$ [29], w_R and w_S are the fitnesses of the resistant and wild-type alleles, respectively, 'log' denotes the natural logarithm, p_R and p_S are the relative population frequencies of the resistant and wild-type allele with $p_R = 1\text{-}p_S$, and the superscripts denote the generation number. Solving for *s* is straightforward in the case of two alleles, but in the case of more alleles, as for *dhfr*, this equation cannot be directly used to estimate the selection coefficient because it gives the fitness of one allele relative to all other alleles combined. In this case, the expected slope for allele *i* ($slope_i$) becomes $\log\left(\frac{w_i(1-p_i)}{\sum_j p_j w_j}\right)$ giving rise to allelic fitnesses of:

$$w_i = \frac{e^{slope_i} \sum_j p_j w_j}{1-p_i} = 1 + s_i \qquad (2)$$

with i = 1,2,3 for single/WT, double and triple mutants, respectively, and where j denotes all alleles other than i. After setting $w_1 = 1$, Equation (2) can be solved for w_2 and w_3 by substituting in the observed values for p_i and p_j and minimizing the sum of squared differences between the observed $slope_i$'s (i.e., those obtained by logistic regression) and their expected values, (i.e., those in Equation (2)). Note, however, that w_i now depends on the frequencies of the other alleles: since these change with time, Equation (2) is an approximation [30]. In the case of *dhfr* in this study, the single and double mutants changed relatively little with time and so the approximation is likely to be accurate.

Confidence intervals (95%) on estimates of *s* were calculated by taking the upper and lower CI values of the regression estimates of the slope and then transforming them using Equation (1). Significance levels of differences between rates of decline of different alleles between different time periods, and when including vs. excluding post-treatment samples, were tested by *t*-tests. data from a previously published study in Malawi [3] were analysed in the same manner. However, in that study, the frequencies of resistant mutants were calculated as the proportion of all samples with both single and mixed genotypes that carried the resistant allele, either alone or with wild-type alleles. Thus estimates of population allele frequency of the mutants in that study are over-estimates.

Results

Four hundred and six, 240 and 323 isolates were genotyped for *pfcrt-76*, *pfmdr1–86* and *dhfr*, respectively, collected between 1993 and 2006 in Kilifi. Of these, 41, 28 and 58 carried mixed alleles and were not used in the analysis. Data on *pfcrt* genotypes from patients that had been treated with AQ (in 2006) were excluded because post-treatment patients harboured a significantly higher frequency of the *pfcrt-76* mutant than pre-treatment patients (19/19 vs. 19/31, $P = 0.002$). There was also an indication that AL selected against the *pfcrt* mutant allele (frequencies of 6/8 pre-treatment vs. 2/7 post-treatment, P = 0.13). There were no significant effects of any other drugs used in the clinical trials on *pfmdr-86* and *dhfr* allele frequencies. Nor did the inclusion of these post-treatment

samples alter the slope of the regression lines in analyses of time trends in allele frequencies. Nevertheless, for the final analyses of time trends, Data from post-treatment samples were excluded. The total number of samples in these analyses was 322, 169 and 248 for *pfcrt*, *pfmdr* and *dhfr*, respectively, with medians of 29, 20 and 23 per year (Additional file 1).

Pfcrt-76 *and* pfmdr1–86

In Kilifi, the frequency of the *pfcrt-76* mutant significantly decreased (P < 0.001) from around 94% to 63% over a period of 13 years (Figure 1a). This decline was occurring before the official withdrawal of CQ in 1999 (P < 0.05). There was no detectable difference in the rate of decline before and after this time ($P > 0.9$ by test for an interaction between the slope and time period). The estimated fitness of this resistant mutant was 5% lower than that of the wild-type (s = -0.05). In Malawi, the rate of decline after CQ was withdrawn was much faster than in Kilifi (from 85% to 13% in 9 years). The estimated fitness of the resistant mutant in the Malawi study was 12% lower than that of the wild-type (s = -0.12): this was significantly greater in magnitude than that in the Kilifi study ($P < 0.001$, by Student's t-test of the logistic regression slopes).

pfmdr-86 did not decline significantly (P = 0.6) in the Kilifi population (Figure 1b), and had a corresponding small selection coefficient of 1%. On the other hand, in the Malawi population, the frequency of the *pfmdr1* mutant allele decreased slightly ($P = 0.05$) with an estimated selection coefficient of -3%.

dhfr

In Kilifi, the frequency of the *dhfr* double and triple mutants combined was already high (around 80%) at the start of the study (five years before SP became the first-line treatment for uncomplicated malaria in Kenya). Nonetheless, this frequency increased significantly ($P < 0.001$) to above 95% during the study period (Figure 2a). Relative to the wild-type/single mutants, the triple and double mutants combined had an estimated selection coefficient of 7%. In contrast, the change in frequency in Malawi was faster (from 15% to above 80% within nine years) with a selection coefficient of 23% for the double and triple mutants combined. This was significantly higher than that for Kilifi ($P < 0.001$ by Student's t-test of the regression slopes). No isolate had a mutation at 164 codon of *dhfr*.

Analysis of the mutant alleles separately in the Kilifi data showed that the selection coefficients of the *dhfr* Asn51Ile-Cyst59Arg double mutants were 5% and 7%, respectively, and 8% for the triple mutant. The frequencies of the double mutants stayed approximately the same through time because, at the same time as they were gaining ground to the wild-type/single mutant, they were losing ground to the triple mutant (Figure 2b). The values of s found here span the range of estimates from other sites in Africa and South-East Asia [31]. The effect of including mixed infections on estimates of s was negligible. Thus the inability to detect alleles at low frequencies within a patient sample by the genotyping methods used here is unlikely to influence the results.

Prediction of pfcrt, pfmdr1 *and* dhfr *allele frequencies in the future*

Based on the above estimates and Equation (1), it is predicted that, if the current drug pressure were to be maintained, Kilifi isolates will have largely reverted to CQ-sensitive phenotypes (frequency of the *pfcrt-76* mutant less than 10%) by the year 2023, over 20 years after the official removal of the drug in Kenya. This contrasts with the case in Malawi where this level of sensitivity was reached within nine years following CQ withdrawal. In Kilifi, virtually no change is expected in *pfmdr1–86* frequency while in Malawi by 2009, the frequency of *pfmdr1–86* mutant is expected to fall below 10%.

In vitro activity

CQ IC_{50} against laboratory reference strains V1S, W2 (*pfcrt-76* mutant) and 3D7 and M24 (wild type) were analysed and and these IC_{50} values are 158 ± 75, 94.15 ± 16, 6.5 ± 2.3, 15.6 ± 8.6 nM respectively. The results of field isolates are summarized in Figure 3. Depending on their *pfcrt-76* genotypes, parasites can be classified into three distinct groups. The first group, the wild-type group, has IC_{50} values ranging between 1 and 25 nM, with an average of 13 ± 12 nM. The second group is composed of mixed genotype infections (wild-type and mutant), with a mean of 24 ± 10 nM; and the third group of *pfcrt-76* mutants, which forms the majority of isolates, has a mean of 63 ± 90 nM (ranging from 5 to 150 nM). To distinguish CQ-sensitive and CQ-resistant isolates, a cut-off point of 100 nM is generally used [32,33]. However, using this cut-off point, most isolates would be classified as CQ-sensitive, yet they are *pfcrt-76* mutant. Thus, the most accurate cut-off point to use in our setting would be 25 nM (Figure 3). The analysis of *pfmdr1–86* genotype showed that wild-type isolates had lower CQ IC_{50} than mutant (57 ± 50 nM versus 68 nM+87 nM), but this difference was not statistically significant (p > 0.05).

Discussion

The results show that CQ resistance in Kilifi was in decline since the early 1990s despite CQ only being officially withdrawn from Kenya in 1999 [5] when it was replaced by SP. In Kilifi District, this replacement was effective, largely owing to an educational programme for rural drug retailers during the change-over period in 1998 to 2001, which raised the proportion of drug users that purchased adequate doses of the first-line drug over-the-counter

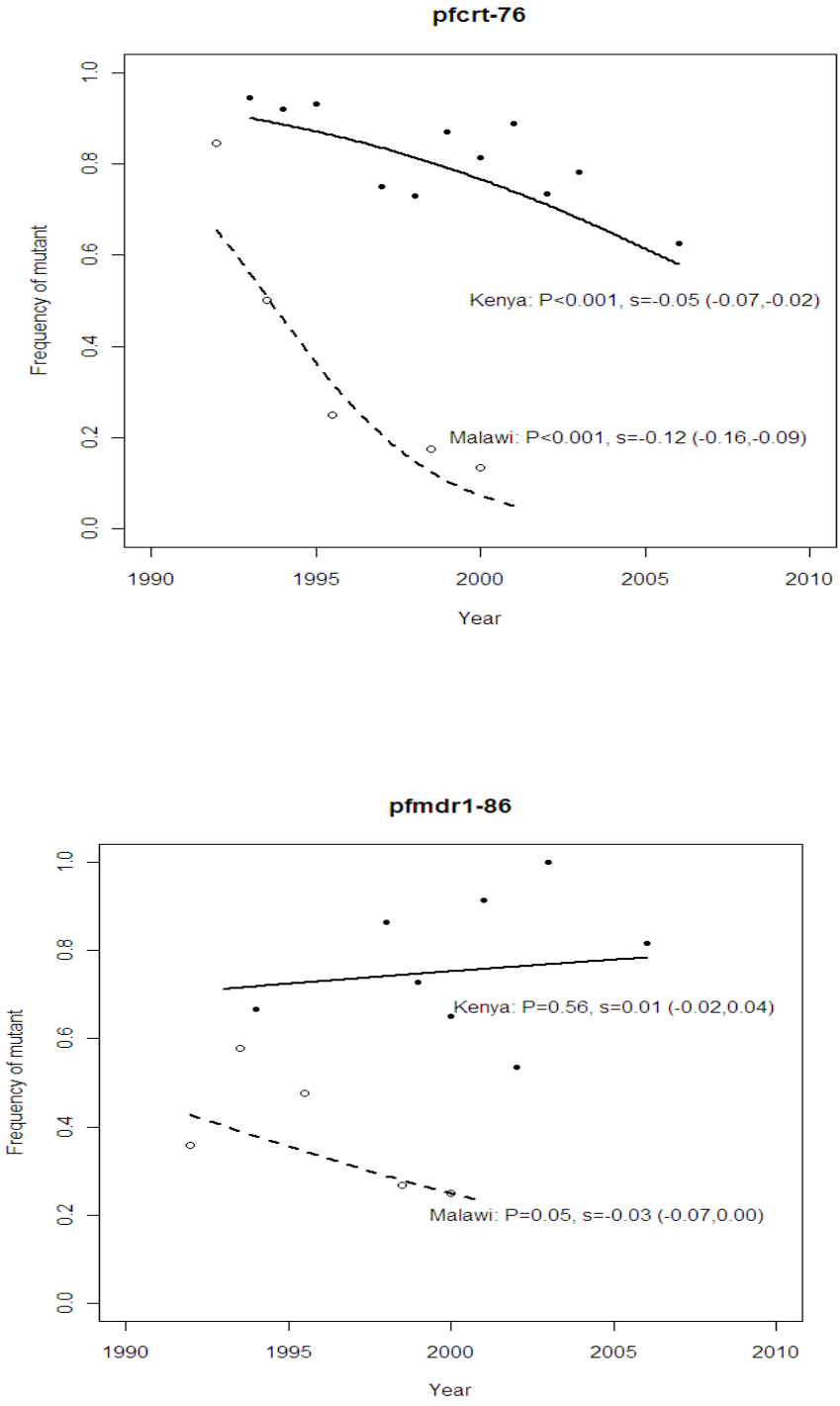

Figure 1
Observed and fitted (by logistic regression) frequencies of *pfrt-76* and *pfmdr1–86* through time in Kilifi, Kenya and a study in Malawi [3]. Observed (symbols) and predicted (lines) population frequencies through time of alleles *pfcrt-76* (top panel) and *pfmdr1–86* (bottom panel) involved in resistance to chloroquine in Kenya (solid line, closed symbols) and Malawi (dashed line, open symbols) before and after the official ban on the use of chloroquine (1999 and 1992, respectively). The *P*-value indicates whether the slope of the predicted line is significantly different from zero. The selection coefficient (s) of the resistant allele relative to the wild-type allele is shown with its 95% confidence intervals for each location.

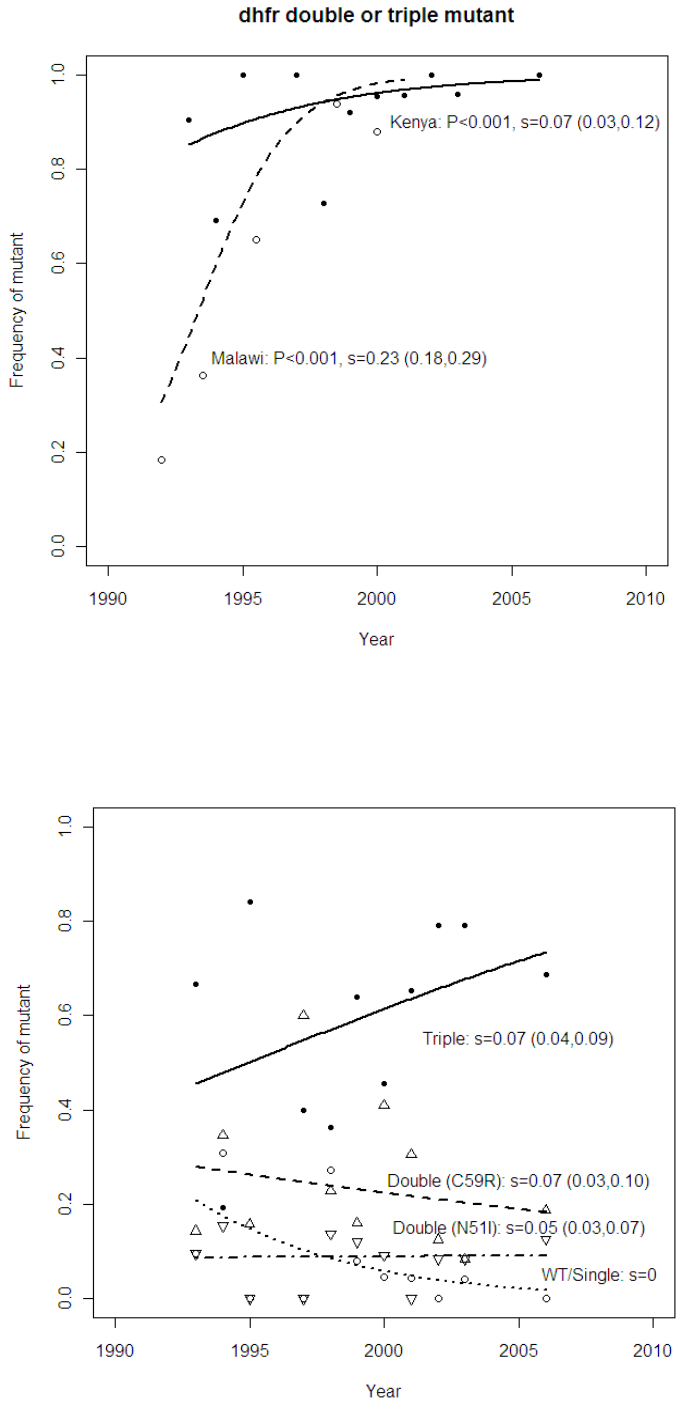

Figure 2
Observed and predicted frequencies of *dhfr* mutations through time in Kilifi, Kenya and a study in Malawi [3]. Observed (symbols) and predicted (lines) population frequencies through time of alleles at the *dhfr* locus determining resistance to pyrimethamine, one of the partner drugs in SP. This drug officially replaced CQ as the first line drug in 1990 and 1992 in Kenya and Malawi, respectively: however, in Kenya, it was used as a second line drug prior to the change from CQ. In the upper panel, the data are for the frequency of the double and triple mutant alleles combined whereas in the lower panel, the alleles are treated separately (see Methods). Selection coefficients are expressed relative to the wild-type/single mutant genotype.

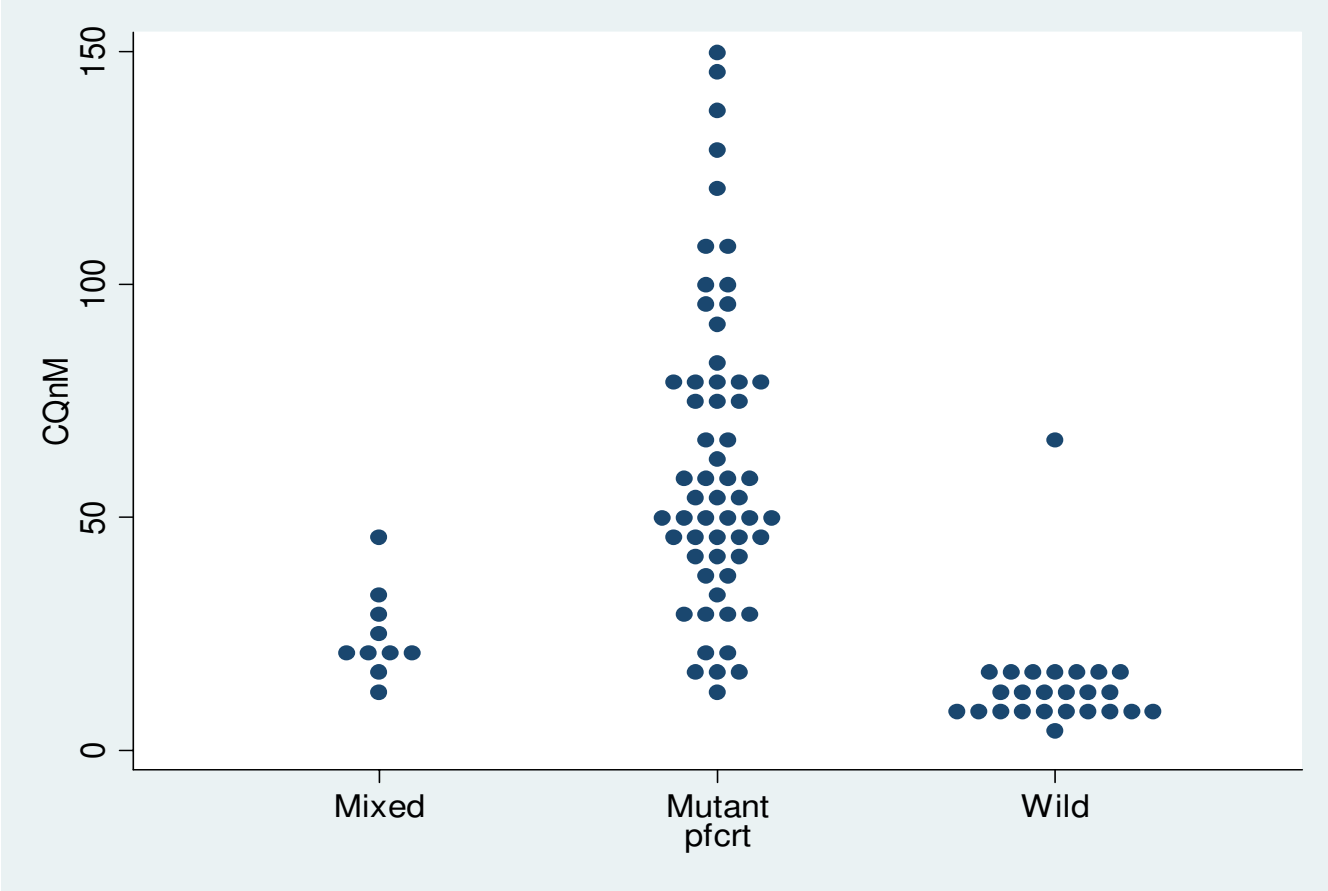

Figure 3
Chloroquine *in vitro* activity and pfcrt-76 genotype. Relationship between chloroquine inhibitory concentration that kills 50% of parasitaemia (IC_{50}) as measured by 50% inhibition of [^{3}H]hypoxanthine incorporation into parasite nucleic acid, and *pfcrt* genotype at codon 76 of isolates collected in 2006 in Kilifi. The dashed line indicates the cut-off level of 25 nM.

(OTC) from 8% to 64% [34]. Thus, following this successful training programme, CQ was largely discontinued in Kilifi, and replaced by a high usage of SP. In other sites in Kenya, however, CQ remained available at the community level as a drug for self-medication, even four years after its withdrawal [35]. The rate of change before and after the point of official withdrawal (2001) was not detectably different ($P > 0.05$).

These results suggest that widespread clinical failure of CQ prior to the ban drove people to use alternative drugs such as SP (which was then the second line of treatment) regardless of the prevailing official policy. This is supported by the high frequency of the *dhfr* double and triple mutants in the Kilifi population from the early 1990s [18] (Figure 2). Furthermore, prior to 2001, even though CQ was the most used anti-malarial, in most cases this drug was used at sub-therapeutic doses [34]. Thus it seems that declining usage and low efficacy due to high rates of clinical failure led to a decline in resistance well before its official discontinuation.

The fact that the frequency of the mutant allele nevertheless declined before the ban during continued use of the drug implies that there is a considerable cost of the *pfcrt* mutation to the parasite in the absence of drugs as even a small amount of drug pressure is sufficient to counteract quite high fitness costs [28]. That this is the case is evident from the 12% lower relative fitness estimated for Malawi after the drug was completely withdrawn. The apparently smaller relative fitness cost of 5% in Kilifi supports our view that the drug also remained in circulation despite the ban, although we consider alternative explanations below.

The frequency of the *pfmdr1* mutant did not significantly decrease over time, and this could be explained by the marginal role *pfmdr1* plays in CQ-resistance compared to

pfcrt [36,37]. This is supported by the results of this study showing that the *pfmdr1* mutants do not have higher CQ IC_{50}s than wild type. However, in Malawi, over few years, the frequency of *pfmdr1* mutants significantly decreased, and this may be due to a rapid decline of CQ-resistance. Thus in Kenya, this may be observed when the *pfcrt* wild type reaches a high frequency in the population.

It is well established that mutations that render an organism resistant to a drug are likely to be associated with a loss of fitness. As a result, organisms carrying these mutations will be outgrown by drug-sensitive organisms when the drug pressure is removed or reduced [38-42]. An increase in CQ *in vitro* activity and a decrease in prevalence of *pfcrt-76* was also observed after the withdrawal of CQ in China [43,44]. Selection of CQ resistance was associated with a loss of fitness in murine malaria, and the same phenomenon has been reported for other anti-malarial drugs, such as pyrimethamine and atovaquone in *P. falciparum* [45-47]. In some instances, loss of fitness may be associated with the development of compensatory mechanisms, leading to persistence of the mutant parasite in the population despite the discontinuation of the drug. This feature may explain, at least in part, the persistence of *pfcrt-76* mutant in parts of South-East Asia and South America [48-50], and in Kilifi as described here, despite CQ not having been used in these areas for many years.

The apparent difference between Kenya and Malawi in the rate of disappearance may also have been due to different usages rates of AQ, a close analogue to CQ. This and other studies have demonstrated that the use of AQ can select for the *pfcrt*-76 mutant [51]. AQ has been the second line of treatment in Kenya since SP was introduced in 1999 and has remained so until now. This drug has partially been used in several sites in Kenya [35], including Kilifi, even before the withdrawal of CQ [34].

CQ *in vitro* activity of Kenyan isolates collected in 2006 in this population were also tested. The data showed that the cut-off point of 100 nM [33] commonly used to distinguish CQ-resistant isolates from CQ-sensitive ones is not appropriate in our setting. We propose that 25 nM is a more suitable cut-off point to use in Kilifi, which is much lower than in other areas. The most likely explanation of this low cut-off point is the variability of the chemosensitivity assay in different settings due to the many factors that influence it [32,52]. For example, here we used the CQ-resistant strains V1S and W2 and the CQ-sensitive M24 and 3D7 as controls. The observation of previous reports show that CQ IC_{50} against these strains are 0.7 time lower to four times higher than IC_{50} values we obtained, and in general, our IC_{50}s against the CQ-resistant strains V1S and W2 were at least 1.5 time lower [32,53-59]. Thus, whilst the *in vitro* chemosensitivity assay is a robust technique to discriminate between CQ-sensitive and CQ-resistant isolates within a setting, there is a need to define the cut-off point in every research setting [32,60].

Conclusion

The current report demonstrates that CQ-resistance has been decreasing in Kilifi since its withdrawal in 1999, but that this decline was much slower than in Malawi. It will take 13 more years for the clinical efficacy of CQ to be restored in Kilifi. The most likely reason for this difference is that the change of drug policy from CQ to SP in Malawi was swift and effective, such that SP became the only available anti-malarial drug for treatment within one year of implementaiton. In Kenya, it appears that the ban was both too late and was ineffectively applied, so that it gave rise to the use of other drugs such as AQ, as well as continued use of CQ, thereby maintaining selection for CQ resistance. Thus, while the highly efficient implementation of drug policy in Malawi that permitted a faster reversal of CQ resistance also promoted more rapid selection for resistance against the replacement drug, SP, than in Kenya, the less rigorous enforcement of drug policy in Kenya has resulted in both the high maintenance of CQ resistance as well as high SP resistance. Thus the effectiveness of drug policy implementation can have important and far-reaching effects on the useful life of life-saving drugs. The newer policies of using drugs in combinations in order to prolong the resistance-free period is a good example of this [28,61].

Abbreviations

CQ: Chloroquine; *dhfr*: dihydrofolate reductase; SP: sulphadoxine/pyrimethamine; Lapdap™: chlorproguanil/dapsone; Artekin®: piperaquine-dihydroartemisinin; AQ: amodiaquine; AL: artemether/lumefantrine; Lys: lysine; Thr: treonine; Asn: asparagine; Tyr: tyrosine; IC_{50}: concentration required for 50% inhibition of [^{3}H] hypoxanthine incorporation into parasite nucleic acid; WT: wild type; OTC: over-the-counter.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LM carried out parasite adaptation, drug chemo-sensitivity assays and genetic analysis. EO contributed in the genotyping of pfcrt, pfmdr1 and dhfr genes. AA and SM contributed in the *in-vitro* culture and drug sensitivity assays. SW, GK, KM and AN designed the study and were involved in manuscript development. PS and SB contributed in generating isolates that were used for the assessment of CQ activity. MM carried out statistical analyses, and contributing in manuscript development.

Additional material

Additional file 1

Numbers of genotypes carrying the mutant form (out of total number of genotypes) after excluding mixed infections and post-treatment samples. *Supplemental table.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1475-2875-8-106-S1.doc]

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In vitro selection of *Plasmodium falciparum* drug-resistant parasite lines

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The *in vitro* selection of antimicrobial resistance in important pathogens can provide critical information on the genetic basis of drug resistance, and such information can be used to predict, anticipate and even contain the spread of resistance in clinical practice. For instance, the discovery of the role of *pfmdr1* in mefloquine resistance in malaria parasites resulted from *in vitro* studies. However, the *in vitro* selection of resistance is difficult, challenging and time consuming. In this review, we discuss the key parameters that impact on the efficiency of the *in vitro* selection of resistance, and propose strategies to improve and streamline this process.

Keywords: 'accelerated resistance to multidrug', clones, per-parasite resistant frequency, mutation rate, resistance indexes

Introduction

The control of malaria rests on the use of mosquito vector control and antimalarial chemotherapy. However, the latter strategy is hampered by the emergence of antimalarial resistance. Currently, there is no antimalarial drug that malaria parasites have not developed resistance against. Worryingly, there is evidence that malaria parasites in Cambodia are becoming resistant to artemisinin derivatives,[1] providing a potential threat to artemisinin-based combination therapy (ACT), the cornerstone of current treatment. This emphasizes the need to reduce drug resistance selection pressure by withdrawing monotherapies,[2] to develop new and affordable drug combinations, and to understand the mechanisms that underlie resistance to existing antimalarials. This could lead to: (i) the development of simple means of tracking the selection and spread of resistance; and (ii) the design and discovery of new and more potent antimalarials.

To study mechanisms of resistance, one needs to obtain well-characterized drug-resistant strains. However, such strains are not generally available for most antimalarials. Murine malaria—*Plasmodium berghei*, *Plasmodium chabaudi*, *Plasmodium yeolii* and *Plasmodium vinckei*—have been used as surrogates for *Plasmodium falciparum* to study the mechanisms of drug resistance by inducing resistance *in vivo*. This approach has led to the selection of drug-resistant parasite lines and subsequent studies on mechanisms of drug resistance.[3] However, for drugs such as chloroquine and probably artemisinin, mechanisms of resistance in murine and *P. falciparum* malaria are different, highlighting the limitations of the murine malaria model.[4–8]

The development of the *in vitro* system for the routine culture of *P. falciparum* in 1976 provided a tool to induce resistance *in vitro*.[9] In 1978, Nguyen-Dinh and Trager[10] reported for the first time the selection of chloroquine resistance *in vitro*. This study heralded a new era and showed that selection of antimalarial resistance could be achieved *in vitro* in *P. falciparum*, thus permitting the study of the mechanisms of antimalarial resistance even before drugs were used in clinical practice. While this approach has been used to select some drug-resistant strains (see Table 1), it has not yet been used to study most of the antimalarials that are either in clinical use, such as the artemisinin derivatives, lumefantrine and piperaquine, or drugs in the pipeline and likely to be used in the near future, such as tafenoquine and pyronaridine.[1,11–13]

Among these drugs, artemisinins are the most important. Indeed, this drug family is currently the backbone of malaria therapy as they form the basis of ACTs, and artesunate is replacing quinine in the treatment of severe malaria.[14–16] A major concern is that resistance to this drug family is now emerging in South East Asia. Strategies are being put in place to predict, control and contain the emergence of artemisinin resistance.[1,17] These strategies would be enhanced if the mechanism of resistance was identified and a simple marker developed for epidemiological studies. PfATPase6 has been proposed as the molecular target and polymorphisms have been associated with artemether resistance,[18] but this has not been confirmed elsewhere.[1] Consequently, in the absence of well-characterized drug-resistant strains, the mechanism of artemisinin resistance still awaits characterization. Could the *in vitro* culture system permit the selection of stable resistant parasites to these important antimalarials?

Table 1. Summary of main findings of *in vitro* selection of resistance to antimalarials in *P. falciparum* strains

Drugs	Strains (drug resistance profile[a])	Ri[c] (IC_{50})	Ratio of HDC[d] per IC_{50} after drug pressure	Time required to select resistance (months)	Stability: period of drug-free culture (months)	Work carried out on parasite lines: main finding	Ref.
CQ	FCR3 (CQ and CG resistant)	NM	NM	4	1	NM	10
	FAC8 (CQ resistant)	2.34 (83 ng/mL)	1.23	NM	1	deamplification of *pfmdr1* in association with CQ resistance	34
	HB3 (PM resistant)	1.64 (28 ng/mL)	1.74	30	NM	DNA amplification in chromosomes 3 and 12; deamplification in chromosome 3 after drug removal	39
	106/01 (CQ susceptible)[b]	12 (37 nM)	0.23	2	NM	evidence that the presence of pre-existing mutations in *pfcrt* lead to a rapid selection of the key 76 mutation	38
MFQ	FCK (CQ resistant)	16 (8 nmol/L)	0.5	3	NM	NM	27
	Smith (CQ, PM and SD resistant)	3.4 (3.5 μg/L)				inverse relationship between MFQ and CQ	28
	Camp (CQ susceptible)	2.4 (4.9–12 μg/L)	1.66	>1[e]	6 and cryopreservation		
	W2 (CQ, PM and SD resistant)	4.6 (4.5 nM)	1.93	22.4	12	1. inverse relationship between CQ and MFQ activity 2. identification of *pfmdr1* as MFQ resistance marker	21
	K1 (CQ, PM and SD resistant)	4.07 (22.4 ng/mL)	0.8	NM	NM	1. MFQ resistance associated with pfmdr1 overamplification	29
	W2mef (CQ, PM, SD and MFQ resistant)	1.41 (58.88 ng/mL)	1.08	NM	NM	2. evidence of inverse relationship with CQ	
	W2mef (CQ, PM, SD and MFQ resistant)	1.07 (15.2 ng/mL)	148.1	18	NM	evidence of cross-resistance with HFT and QN and inverse relationship with CQ	26
HFT	T9.96 (CQ susceptible)	3.3 (6.6–22 nM)	0.45	6	6 and cryopreservation	cross-resistance with QN but inverse relationship with CQ	71
	K1 (CQ and SD resistant)	9 (2.2 nM)	0.4	2			
PM	FCR3	NM (15 nM)	NM	7		DNA amplification (chromosome containing *dhfr*[f])	40
5FO	W2	100 (2 nM)	1	2	NM	evidence that resistance emerges quicker in already resistant strains	63
	FCR3	NM	1	2	NM		
ATV	W2	30 (3 nM)	1.1	2	NM	evidence that resistance emerges quicker in already resistant strains	63
	K1	837 (13.6 nM)	1.6	NM	<3	evidence that mutations in cytochrome *b* are associated with ATV resistance	46
BMS-3888891	Dd2 (CQ, QN, PM and SD resistant)	12 (10 nM)	NM	2.66	NM	evidence that resistance is associated with point mutation in protein farnesyl transferase	51

Continued

Table 1. *Continued*

Drugs	Strains (drug resistance profile[a])	Ri[c] (IC_{50})	Ratio of HDC[d] per IC_{50} after drug pressure	Time required to select resistance (months)	Stability: period of drug-free culture (months)	Work carried out on parasite lines: main finding	Ref.
N-89	FCR3	10 (25 nM)	NM	24	NM	no cross-resistance between the endoperoxides N-89 and artemisinin	70
AZ	Dd2	15.3 (124 nM)	NM	0.7	NM	AZ resistance is associated with point mutation in ribosomal protein L4 (pfRpL4)	59
	7G8 (CQ and PM resistant)	17.5 (228 nM)	NM	0.7	NM		

CQ, chloroquine; CG, cycloguanil; SD, sulfadoxine; PM, pyrimethamine; HFT, halofantrine; MFQ, mefloquine; ATV, atovaquone; 5FO, 5-fluoro-orotate; AZ, azithromycin; QN, quinine; NM, not mentioned.

[a]Information on resistance phenotype was presented in the references listed in the table and in Nkrumah *et al.*[71]

[b]This strain is CQ susceptible; it has mutations in five codons of *pfcrt*, but not at codon 76. After drug pressure, two parasite lines were obtained: one with pfcrt-76 asparagine and a second with pfcrt-76 isoleucine, with IC_{50} values of 302.2 and 443.1 nM, respectively. We used the highest IC_{50} (443.1 nM) in the table. The normal mutant in CQ resistance is pfcrt-76 threonine.

[c]Ri, resistance index: the ratio of the IC_{50} of the selected parasite line to the IC_{50} of the parent strain (before drug pressure).

[d]HDC, highest drug concentration tested, in which the parasite lines could grow after drug pressure.

[e]Exact time period was not given.

[f]*dhfr*, dihydrofolate reductase gene.

The selection of *in vitro* resistance is very slow; it can take months or even years for a stable drug-resistant line to emerge. This limitation combined with the difficulties of the *in vitro* culture technique (risk of contamination of cultures and slow parasite growth) might explain why for many antimalarials no stable resistant strains have been reported up to now. A recent review has presented the methods and application of the *in vitro* and *in vivo* selection of resistance.[19] In the current paper, we have reviewed work carried out on the *in vitro* selection of resistance for the past 30 years. This review has identified the key parameters that are critical in the success of this process. We propose strategies to streamline the *in vitro* selection of antimalarial drug resistance.

First and benchmark work

The first work on the selection of *P. falciparum in vitro* was reported in 1978 by Nguyen-Dinh and Trager[10] using the then newly developed method of malaria culture, the Petri dish method.[9] A Gambian (West Africa) strain (FCR3), whose growth was completely inhibited at a concentration of 100 ng/mL chloroquine, was grown in the presence of increasing chloroquine concentrations, starting from 10 ng/mL. After 15 cycles (2 months), a parasite line that could grow in the presence of 100 ng/mL chloroquine was selected. This resistant phenotype was stable, since the parasite could grow in drug-free medium for several weeks without losing the selected phenotype. This work was perceived as a landmark, as it opened up the possibility that resistance could be selected against antimalarials *in vitro*.

How to interpret the data on the *in vitro* selection of resistance

We focused our review on publications that provided sufficient information on the *in vitro* selection experiment, including IC_{50} values (concentration that inhibits 50% of parasite growth), the protocol used and stability information. We reviewed 16 publications and summarize the results in Table 1.

Right shift of the dose–response curve

In vitro, drug activity is assessed by growing the parasite in the presence of various drug concentrations (serial dilution). The equation for the parasite growth rate as a function of the drug concentration generates a sigmoid dose–response curve, from which are deduced IC_{50}/IC_{90} values (inhibitory concentrations that inhibit 50% or 90% of parasite growth). Resistance is defined by a right shift of the curve (Figure 1). The shape of the shift can be parallel, with the same maximum parasite killing effect (E_{max}) as the parent strain, or this shape could change, with a reduced maximum parasite killing effect (Figure 1).[20]

We define the resistance index (Ri) as the ratio of the IC_{50} of the resistant line to that of the parent strain. Thus, the higher the Ri, the higher the level of resistance. However, a low Ri does not necessarily mean a low level of resistance. As shown in Table 1, Ri values can vary from 1 to >800. It is clear that an Ri of 100 indicates high-level resistance (found on strain W2 after drug

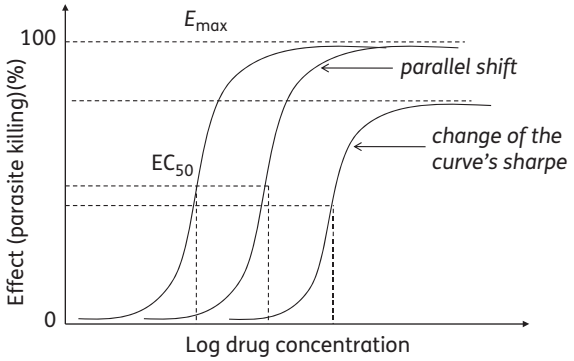

Figure 1. Drug–response as a function of the concentration. Any right shift of the curve denotes an increase in the IC_{50} and, thus, the resistance. The shift can be parallel or the shape of the curve and the maximum effect (E_{max}) could change. Reproduced from *Trends in Parasitology*, 18(10), White NJ, The assessment of antimalarial drug efficacy, 458–64, 2002, with permission from Elsevier.[20]

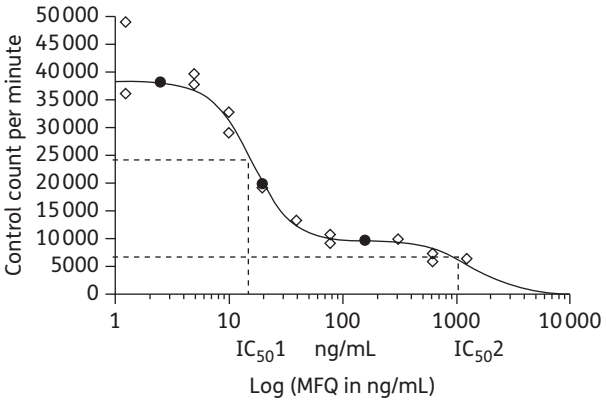

Figure 2. Dose concentration–response of an *in vitro*-selected mefloquine (MFQ)-resistant line that could grow in the presence of >1200 ng/mL mefloquine. The dose–response was analysed using a modification of the logistic logarithmic function that permits evaluation of a biphasic concentration–response relationship. Two parasite populations with different IC_{50}s can be defined from this graph, yet the use of a monophasic concentration–response would have generated one single IC_{50} (inhibitory concentration that kills 50% of parasites). Reproduced, with permission, from Peel *et al.* (Figure 4b).[26]

pressure of atovaquone and 5-fluoro-orotate) (Table 1). An Ri of <10 may indicate an intermediate resistance level. However, it is interesting to note that the mefloquine-resistant parasite line (from W2), which was used to identify the mechanism of mefloquine resistance, had an Ri of only 4.6 (Table 1), implying that even a small right shift of the curve could be associated with resistance.[21,22] *In vivo*, the relationship between the degree of resistance and the resulting therapeutic responses is complex, and depends on parameters such as the host immune response and the pharmacokinetic and pharmacodynamic properties of the drug.[68]

Variation of in vitro assay data

There is interlaboratory (interassay) variation of IC_{50}/IC_{90}. The culture conditions, mainly the initial parasitaemia, haematocrit, the time of incubation, the timepoint when the label is added (hypoxanthine for instance), the use of normal or substitute serum and the gas mixture composition, have been singled out as influencing the test.[23,24] A right shift as a result of interassay variation could be wrongly interpreted as reflecting resistance. Therefore, any right shift is considered valid only if all *in vitro* culture parasite parameters are maintained constant.

Instability of the resistant phenotype

The resistant phenotype is stable when it remains unchanged after growing parasites in drug-free medium. In Table 1, in studies in which stability was reported, parasite lines remained resistant when grown in the absence of the drug during time periods varying from 2 weeks to 1 year and, in two studies, resistance remained unchanged after parasite cryopreservation. The unstable phenotypes could be associated with reduced parasite fitness, explaining why, once drug pressure is removed, the phenotype reverts to normal. For instance, a recent study has shown that parasites grown under mefloquine pressure expressed multiple *pfmdr1* copy numbers and that these parasites had a significantly decreased survival fitness compared with parasites with a single *pfmdr1* copy number.[25]

Existence of more than one parasite population during drug pressure

We computed the ratio of the highest drug concentration at which the parasite grew normally during the selection process with the parasite drug IC_{50} after drug selection (highest drug concentration tested/IC_{50}, Table 1). This ratio is expected to be <1, since it represents the ratio of a subinhibitory concentration (thus, $<IC_{50}$) divided by the IC_{50}. In 7/15 strains, this ratio was ≥1.1. The most striking data were observed with W2mef on mefloquine,[26] showing a ratio >148. This indicates that this parasite line could grow in the presence of a drug concentration 148 times higher than its own IC_{50}. This led to the discovery that, in fact, there were two parasite populations with different IC_{50} values—one with a lower value, which corresponds to the dominant population, and another minor population, with a higher IC_{50} value. The second population is the one that persists when parasites are cultured in the presence of high drug concentrations. When determining IC_{50} values, drug pressure is removed and, because of the fitness disadvantage conferred by the drug-resistant phenotype, the minor population is replaced by the dominant and drug-susceptible one, reflecting low IC_{50} values. Since the determination of IC_{50} values is based on the use of a 'monophasic' drug–response curve, only one IC_{50} is obtained, which represents the dominant population. However, the presence of two populations in a mixture can be identified using a 'biphasic' drug–response curve.[26] Figure 2 summarizes the graphic representation of the use of a biphasic drug–response curve leading to the identification of two populations.[26] Thus, a ratio of >1 indicates the existence of at least two parasite populations and selection emerges on the backdrop of the minor population, which gradually becomes dominant as the drug-pressure experiment continues.

How useful has *in vitro* resistance been?

The main objective of inducing *in vitro* resistance is to generate parasite lines that can be used to study the mechanism of resistance. How much has already been done with the *P. falciparum* cell lines that were selected *in vitro*?

Mefloquine and chloroquine

The first selection of *in vitro* mefloquine resistance was carried out in the early 1980s.[27,28] In 1988, Oduola *et al.*[21] selected the mefloquine-resistant strain W2mef, from the parent strain W2. The analysis of P-glycoprotein *pfmdr1* between W2 and W2mef led to the discovery of the association between amplification of this gene and mefloquine resistance.[22] Thereafter, the WEHI group (Walter and Eliza Hall Institute) induced mefloquine resistance against the K1 strain and further against the W2mef line, and confirmed the involvement of *pfmdr1* in mefloquine resistance.[29] Since then, these findings have been supported in field isolates[30–33] and in rodent malaria.[5]

The aforementioned studies and the one by Barnes *et al.*[34] on the selection of chloroquine resistance (Table 1) showed the existence of an inverse relationship between the *pfmdr1* copy number (mefloquine resistance) and chloroquine susceptibility. This prompted further investigations into *pfmdr1* polymorphism in relation to chloroquine susceptibility. Several single nucleotide polymorphisms in this gene have been associated with chloroquine resistance and it has now been established that this gene plays an ancillary role in chloroquine resistance, the key gene being *pfcrt*.[30,35–37]

The use of *in vitro*-selected chloroquine-resistant parasite lines has also led to a better understanding of the mechanisms of chloroquine resistance. Chloroquine resistance was induced in the Sudanese strain 106/1.[38] This strain contains seven mutations associated with chloroquine resistance in *pfcrt* (at codons 74, 75, 220, 271 and 371), but the key one, at codon 76, is wild type (lysine), while chloroquine-resistant isolates have threonine at this codon. This strain was chloroquine susceptible. By subjecting it to chloroquine pressure for 2 months, a chloroquine-resistant line was selected. Subsequent genetic analyses demonstrated that this selected chloroquine-resistant phenotype was associated with the presence of a mutation at *pfcrt* codon 76 (from lysine to isoleucine or asparagine). Though this mutated amino acid was not threonine, this study confirmed the critical role of the mutation at codon 76 in chloroquine resistance.

The HB3 strain was used to select chloroquine resistance and further analysis demonstrated the existence of DNA amplification in chromosomes 3 and 12 in this parasite line.[39] It is interesting to note that neither *pfmdr1* nor *pfcrt* are located on chromosomes 3 or 12. Since the mechanism of chloroquine resistance is known to be multifactorial, other genes might be involved; however, no further studies have been carried out on these cell lines.

Pyrimethamine

Banyal and Inselburg[40] selected FCR3 parasite lines resistant to pyrimethamine. This phenotype was found to be associated with DNA amplification on chromosome 4 in a region corresponding to the dihydrofolate reductase–thymidine synthase gene (*dhfr-ts*).[41,42] It was also found that these parasite lines harbour a point mutation at codon 223 of the *dhfr* domain,[42] in line with the role of this gene in pyrimethamine resistance.[43–45]

Atovaquone

This drug has been combined with proguanil to form Malarone®, one of the most potent antimalarial prophylactic agents. Atovaquone binds to cytochrome *b* (Cyt b), leading to the inhibition of mitochondrial electron transfer, and mutation in Cyt b has been associated with atovaquone resistance *in vivo*.[46] Using drug pressure on the K1 strain, Korsinczky *et al.*[46] selected parasite lines resistant to atovaquone, with Ri varying from 76- to 837-fold. Sequencing of the Cyt b gene of these parasites showed point mutations at codons 275, 272, 280, 281, 284 and 280, and these codons are located in the binding pocket of atovaquone to Cyt b.[46] Interestingly, the mutation at 268, which is commonly found in atovaquone-resistant field isolates, is also located in this binding pocket.[47–49]

BMS-388891

This compound is an inhibitor of protein farnesyl transferase (PFT), which catalyses the transfer of the farnesyl group to the C-terminus of a specific set of proteins, including those that are essential in cell multiplication, and the blockade of this farnesylation is associated with cell death.[50] Inhibition of the PFT enzyme is a good drug target against malaria, and many inhibitors have been synthesized and evaluated as potential antimalarials.[50–52] Eastman *et al.*[51] induced resistance to an inhibitor of PFT, BMS-388891, by culturing the Dd2 strain in the presence of continuous drug pressure. Within 80 days, a line with a 12-fold decreased activity was selected and genetic analyses have shown the existence of a point mutation at codon 837 of PFT. Thus, polymorphism in the PFT enzyme could be associated with *in vivo* resistance to inhibitors of PFT in the clinical setting.

Azithromycin

The macrolide azithromycin, along with erythromycin, has proved to be active against *P. falciparum*, and several clinical trials of azithromycin and erythromycin alone or in combination with chloroquine, quinine or artesunate have been conducted. These macrolide agents are promising antimalarials, especially in the treatment of malaria in pregnancy.[53–58] An azithromycin-pressure experiment on Dd2 and 7G8 strains led to the selection of resistant lines within 21 days, and these lines showed cross-resistance to erythromycin.[59] This phenotype was associated with point mutations in *P. falciparum* apicoplast-encoded ribosomal protein L4 (pfRpL4), one of the proteins that form the 50S ribosomal subunit,[59] in line with the macrolide resistance mechanism in bacteria.[59] Though azithromycin and erythromycin are still experimental drugs, the use of laboratory-induced resistant lines has led to the identification of drug resistance genes before these drugs enter clinical use. The ease with which *in vitro* resistance to macrolides has been selected *in vitro* may indicate that the selection and spread of *in vivo* resistance may be rapid. This drug is still in clinical evaluation against malaria

and it remains to be seen whether this prediction will be confirmed *in vivo*.

This information clearly demonstrates the value of drug-resistant parasite lines selected *in vitro*. For instance, *pfmdr1*, one of the genes central to resistance to quinoline or quinoline-related drugs, was identified using *in vitro*-selected drug resistance parasite lines. The use of the same approach has led to the confirmation of pyrimethamine and chloroquine mechanisms of resistance. Work on the macrolide azithromycin and an inhibitor of PFT has demonstrated that it is possible to understand the mechanism of drug resistance before the drug is used in the clinical setting, and before resistant parasites are found in the field.[51,59] In theory, the same could be achieved with any other antimalarial drugs.

Are there strains more prone to becoming drug resistant?

Early reports using murine malaria indicate that a strain resistant to one drug is more amenable to give rise to resistant lines to another drug, compared with strains that are fully drug susceptible. Pyrimethamine- and chloroquine-resistant parasites were more easily generated from drug-resistant strains than from drug-susceptible ones in *P. chabaudi* and *P. vinckei*.[60–62] Evidence that the same phenomenon prevails in *P. falciparum* has been provided by Rathod's group.[63] They induced resistance to atovaquone and 5-fluoro-orotate against the multidrug-resistant strain W2 (cycloguanil, pyrimethamine and sulfadoxine resistant), FCR3 (pyrimethamine and cycloguanil resistant), HB3 (pyrimethamine resistant), 3D7 (sulfadoxine resistant) and the fully drug-susceptible D6. They demonstrated that W2 was more prone to generate drug-resistant parasite lines than FCR3 and that the latter was more prone than 3D7. Not a single resistant line was obtained from the fully drug-susceptible D6. The W2 strain acquired resistance to these drugs with a frequency 10- to 1000-fold higher than the frequency in other strains. This phenomenon was named 'accelerated resistance to multidrug (ARMD)' and is different to the known multidrug-resistant phenotype.[63] ARMD is characterized by the ability of a strain to generate a drug-resistant clone when put under drug pressure. This results from the high mutation rate during parasite multiplication. The low efficiency of the mechanisms of DNA repair has been proffered as one of the factors that contribute to this ARMD phenotype.[19,64]

For instance, HB3 is susceptible to almost all antimalarial drugs, except pyrimethamine. It has one single point mutation at codon 108 in *dhfr*[65] and, thus, has a low level of pyrimethamine resistance. As shown in Table 1, chloroquine drug pressure for 30 months gave rise to a line with an Ri of only 1.64, a value that is among the lowest in Table 1. On the other hand, high level of drug-resistant phenotype does not necessarily imply a higher ability to generate drug-resistant lines. This is the case for W2mef. This parasite line is resistant to many antimalarials and is, thus, more prone to generate resistant clones (as discussed earlier). However, it could not generate resistant clones when subjected to mefloquine drug pressure (Ri <2, see Table 1).[26,29] The reason could be that it was already mefloquine resistant, as a result of *pfmdr1* being overexpressed. W2mef, therefore, needed to develop new and additional mechanisms to cope with the higher mefloquine concentration, explaining, at least partially, the difficulties in generating stable clones with higher mefloquine resistance. This is supported by the fact that, *in vivo*, no new mechanisms of mefloquine resistance have been reported other than *pfmdr1* amplification. Thus, the ability of a parasite to generate resistant lines will vary from one strain to another.

What should be considered when designing an '*in vitro* drug selection' study?

The following are critical parameters that have to be considered when setting up the experiment.

Initial strains

Strains are different; there are those that are more prone to generate resistant parasite lines than others, and these are called ARMD strains (see previous section). Thus, *in vitro* selection for resistance should focus on drug-resistant strains/isolates.

Initial parasitaemia

One of the key parameters in the success of these experiments is the initial parasitaemia. Work in animals has shown that the higher the initial parasitaemias, the higher is the chance of selecting drug-resistant mutants. For instance, Ramakrishnan *et al.*[66] could select sulfadiazine resistance in *P. berghei* in mice at 10% parasitaemia, but failed to do so at 1%. The most interesting evidence was provided in humans, where it was shown that the chance of developing pyrimethamine resistance after a single-dose treatment was associated with the size of the parasite population.[67]

Genetic events that confer *de novo* drug resistance (gene point mutations or copy number variations) are spontaneous and rare, and are drug independent. *In vitro*, parasites undergo mitotic nuclear division exclusively. Thus, the chance of obtaining a mutant strain that will confer drug resistance will be a function of the number of mitotic events and, thus, the size of the parasite population.

The generation of *de novo* resistance is also dependent upon the rate at which parasites generate drug-resistant clones. Since this rate, the per-parasite resistance frequency (PRF), varies depending on the mutation (or the gene), different drugs will have different rates; if resistance involves one single gene, the PRF is likely to be higher than when resistance is multifactorial. For instance, *in vivo*, the PRF for pyrimethamine, atovaquone and mefloquine has been estimated to be 10^{11}, 10^{12} and 10^{14}, respectively; chloroquine has been proposed to be $\sim 10^{19}$; and values $<10^{18}$ have been proposed for artemisinin.[68] Interestingly, in general, PRF rates are found to be higher *in vivo*, e.g. atovaquone-resistant mutants arise at a frequency of 1 in 10^5,[63] and the rate with mefloquine is 1 in 10^8 and 1 in 10^{13} when resistance is associated with one and two/three *pfmdr1* copy numbers, respectively.[25] Thus, different drugs will have a different chance to encounter a drug-resistant clone *in vitro*.

There are $\sim 5\times10^{16}$–5×10^{17} circulating parasites in the human population.[2] If such numbers of parasites could be put into a flask to induce *in vitro* resistance, one could expect that

resistance strains would emerge against any given drug, since, in theory, resistant strains would already be present in the population. Generally, cultures are carried out in small flasks (15 mL) containing 5 mL of the culture suspension [2% haematocrit, equivalent to 100 μL of red blood cells (RBCs)]. Assuming that 10% parasitaemia could be reached after culture, only 10^8 infected RBCs could be obtained using such flasks.

For drugs such as atovaquone with a higher PRF, chances are high that a resistant clone will emerge after some mitotic events with a starting parasitaemia of 10^8; thus, under these conditions, atovaquone resistance would be relatively easy to select, as shown in Table 1. On the other hand, for drugs with a low PRF, such as artemisinin, a starting parasitaemia of 10^8 will not be favourable to the selection of resistance. Thus, current experimental conditions *do not* favour the emergence of resistant mutants against drugs with a low PRF. Thus, experiments should be carried out under conditions where a higher parasite number could be used. There should not be a limitation of parasite number, apart from that imposed by the experimental conditions.

Use of current multidrug-resistant strains

One of the characteristics of ARMD strains is that they are multidrug resistant. The more drugs that parasites have been exposed to, the higher their chance of harbouring an ARMD phenotype. The current strains used in selection of resistance studies were collected 20–30 years ago. For instance, W2, the most commonly used strains to induce resistance, were collected in the 1970s. Since then, parasites have been exposed to new antimalarials. It is reasonable to state that the current multidrug strains are more prone to generate resistance than strains collected 30 years ago. Consequently, the selection of resistance should also include contemporary parasite strains to maximize the chance of obtaining drug-resistant clones. Parasites from malaria-endemic areas where drug resistance is high, for instance South East Asia, should be considered. Another approach would be to use genetically modified parasites that are more prone to generate new mutants. Such parasites could be those with impaired DNA repair mechanisms;[64] however, they are not available yet.

Time of experiment

The success of the selection of resistance depends on the length of the experimental time period. The longer the selection of resistance takes, the higher the chance of obtaining resistant lines. In theory, for any drug, resistance will eventually emerge when given sufficient time. The question is therefore how long the process should take or when to stop.

The process can stop for two reasons. The first could be that parasites have reached the limit of acceptable concentration beyond which they can no longer grow. Alternative drug-free and short exposures to high drug concentrations could overcome this limitation.[26] The second reason, which is more common, is that the process is stopped by the experimenter. For instance, two experiments in which the authors failed to select artesunate (an artemisinin derivative) and ferroquine resistance were stopped within 1 month of drug pressure.[6,69] As we discussed, for a drug such as artemisinin, which has the lowest PRF, it would be surprising that resistant parasites would emerge *in vitro* within 1–2 months. Thus, it is reasonable to expect that more time, even in terms of years, would be required to induce *in vitro* resistance against this drug. It is therefore critical that the experimenter bears these considerations in mind when setting up drug resistance studies. For instance, Aly *et al.*[70] selected a parasite line resistant to the endoperoxide N-89 after 2 years of continuous drug pressure. However, this N-89-resistant strain was susceptible to the endoperoxide artemisinin, indicating that the mechanisms of resistance between these drugs are different.

Conclusions

Thirty years ago, the first report that chloroquine resistance could be induced *in vitro* heralded a new era with the expectation that antimalarial-resistant parasite lines could be obtained, allowing study of the mechanism of resistance before drugs reached clinical use. Although the approach has been used to discover and/or confirm the mechanism of resistance to various antimalarials, including mefloquine, chloroquine and pyrimethamine, it is surprising to note that for important drugs such as artemisinin or lumefantrine, which now form the backbone of malaria treatment, no well-characterized resistant parasite lines exist, limiting investigations on their mechanism of resistance. Yet the use of an *in vitro* system could allow the selection of resistant parasites against any antimalarials. Attempts have been made to select resistance to artemisinin *in vitro*, but in vain. The selection of resistance is challenging and time consuming. Our review of the literature shows that strategies exist to streamline this process. With the development of new molecular technologies, such as Solexa Illumina and 454-sequencing, which allow high-throughput DNA sequencing and whole transcriptome analysis, it is now possible to study drug resistance mechanisms by exploring the parasite whole genome variation at a relatively low cost and in a short time period. However, the challenge remains the generation of drug-resistant parasite lines.

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Transparency declarations

None to declare.

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MAJOR ARTICLE

In Vivo and In Vitro Efficacy of Amodiaquine against *Plasmodium falciparum* in an Area of Continued Use of 4-Aminoquinolines in East Africa

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In light of reports of increasing resistance of parasites to amodiaquine in African countries in which *Plasmodium falciparum* is endemic as well as the paucity of recent in vitro sensitivity data, we assessed the in vivo and in vitro sensitivity to amodiaquine of *P. falciparum* isolates from 128 pediatric outpatients (0.5–10 years old) in Pingilikani, Kilifi District, Kenya, who were treated with amodiaquine (10 mg/kg/day for 3 days). The polymerase chain reaction–corrected parasitological cure rate on day 28 (by Kaplan-Meier analysis) was 82% (95% confidence interval [CI], 74%–88%). Twenty-six percent (17/66) of tested pretreatment *P. falciparum* field isolates had 50% in vitro growth inhibition at concentrations of N-desethyl-amodiaquine (DEAQ)—the major biologically active metabolite of amodiaquine—above the proposed resistance threshold of 60 nmol/L, but baseline median DEAQ 50% inhibitory concentration values were not associated with subsequent risk of asexual parasite recrudescence (29 nmol/L [95% CI, 23–170 nmol/L] and 34 nmol/L [95% CI, 30–46 nmol/L] for patients with and those without recrudescences, respectively). The median absolute neutrophil count dropped by 1.3 × 10^3 cells/μL (95% CI, -1.7×10^3 to -0.7×10^3 cells/μL) between days 0 and 28. The high prevalence of in vitro and in vivo resistance precludes the use of amodiaquine on its own as second-line treatment. These findings also suggest that the value of amodiaquine combinations as first- or second-line treatment in areas with similar patterns of 4-aminoquinoline resistance should be reassessed.

Because of rare but serious hematological and hepatic toxicity when used for malaria prophylaxis [1, 2], amodiaquine is recommended only for treatment of falciparum malaria [3, 4]. There was a renewed interest in amodiaquine monotherapy in the face of high chloroquine resistance in the 1990s, because it was shown to retain higher efficacy than chloroquine against chloroquine-resistant parasites [5, 6]. Since then, amodiaquine has been used widely in Africa as an alternative to chloroquine or as second-line treatment. Today, amodiaquine is one of the most important front-line drugs for malaria control in Africa. In combination with artesunate, amodiaquine is used as first-line treatment in 17 countries [7]. In Kenya, amodiaquine was the second-line drug when sulfadoxine-pyrimethamine (SP) was the first-line drug per the national policy (1998–2004) [8] and was used as the first-line drug during the interim (2004–2006) before the implementation of artemether-lumefantrine as first-line treatment [9].

Although recent data reported by the East African Network for Monitoring Antimalarial Treatment (obtained using day 14 cure rates as the primary outcome measure) seem to support the continued use of amodiaquine [10], other in vivo studies from areas in both

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West and East Africa, including Kenya, have reported increasing parasite resistance to amodiaquine [11–13]. There appears to be considerable heterogeneity in the geographical distribution of parasite sensitivity to amodiaquine. For instance, polymerase chain reaction (PCR)–corrected per-protocol treatment failure rates by day 28 in the amodiaquine monotherapy arm of a multicenter trial comparing amodiaquine with artesunate-amodiaquine for the treatment of uncomplicated *P. falciparum* malaria varied from 21% in Senegal and Gabon to an alarming 71% in Entasopia in southern Kenya [14] (M. Loolpapit, unpublished data).

The scarcity of recent in vitro sensitivity data from the East African region limits a comprehensive interpretation of the findings of in vivo studies, especially of those reported by effectiveness trials. One study from Rwanda showed a low prevalence (7%) of in vitro resistance to the major biologically active metabolite of amodiaquine, N-desethyl-amodiaquine (DEAQ) [15]. Comparative chemosensitivity data are also required for the appraisal of new amodiaquine derivatives as they progress through clinical development [16]. The aim of the present study was to determine the current pattern of in vivo and in vitro *P. falciparum* sensitivity to amodiaquine in an area in which the drug has been extensively used since 2003.

METHODS

Study area. The study was conducted at the Pingilikani Clinical Trials facility of the Kenya Medical Research Institute (KEMRI) Center for Geographic Medicine Research–Coast (CGMR-C), which is located 20 km south of the town of Kilifi, on the Kenyan coast. CGMR-C operates this study site adjacent to a primary health care clinic in collaboration with local Ministry of Health authorities. Pingilikani is in the southern part of the area that is demographically surveyed by the KEMRI center and is characterized by high year-round transmission of *P. falciparum* and an estimated entomological inoculation rate of between 22 and 53 infective bites per person per year [17], although the characteristics of transmission in the area are changing [18] (authors' unpublished data). Subsidized insecticide-treated bed nets for children <5 years old and pregnant women are available at the Pingilikani Dispensary, and use is encouraged. The study was conducted during an interim period between a change of policy from sulfadoxine-pyrimethamine to artemether-lumefantrine as the first-line treatment for uncomplicated *P. falciparum* malaria. Amodiaquine has been used as first-line treatment for pediatric malaria case management at the Pingilikani Dispensary since 2003. The study protocol was reviewed and approved by the KEMRI Scientific Steering Committee and the National Ethical Review Committee.

Study design and sample size. The study was designed as a prospective observation of pediatric outpatients attending the Pingilikani Dispensary. Eligible patients received a supervised oral course of amodiaquine targeting a dose of 10 mg/kg/day for 3 days and were subsequently followed up for 28 days. Sample size estimation was based on the accepted practice of using a day 28 PCR-corrected cure rate of 90% (10% failure rate) as the cutoff point for differentiating between effective and failing drug therapies [19]. Our study was designed to detect parasitological failure rates of >10% by a ⩾8% margin with 95% confidence, and, allowing for an estimated lost-to-follow-up rate of 17%, the minimum required sample size was calculated to be 120 patients.

Enrollment of patients. The study was conducted from March through July 2006. Pediatric patients attending the Pingilikani Dispensary were enrolled into the study if they met the following inclusion criteria: a history of fever during the preceding 24 h or an axillary temperature of ⩾37.5°C; age of 6 months to 10 years; weight of ⩾5 kg; microscopically confirmed *P. falciparum* monoinfection with an asexual parasite density of 2000–200,000 parasites/μL; resident in the study area; and provision of written informed consent by their accompanying parent or guardian. Exclusion criteria were as follows: adequate antimalarial intake for the current febrile illness; known hypersensitivity to amodiaquine; symptoms and signs of severe malaria [20]; any other severe underlying disease; concomitant disease masking the assessment of treatment response; danger signs; and severe malnutrition (weight-for-height values <70% of the median given on the National Center for Health Statistics/World Health Organization [WHO] reference tables).

Study drug and administration. Amodiaquine was formulated as 200-mg base tablets (Parke Davis), and the tablets (rounded to the nearest half tablet) were administered orally under direct supervision. Tablets were administered with drinking water; for infants and young children (<2 years old), the tablets were crushed, mixed with 25 mL of drinking water, and administered as slurry. Participants who vomited or rejected the study drug within 30 min received a second full dose, and those who vomited or rejected the study drug after 30 min but within 1 h received a second half dose. Vomiting or rejecting the second dose led to withdrawal from the study and administration of rescue medication (6 doses of oral artemether-lumefantrine over 3 days).

Study flow and procedures. Patients were seen by a study clinician for baseline assessment before administration of the first dose of study medication on days 0, 1, and 2 and again on days 3, 7, and 28 (or otherwise if indicated). Patients who failed to return for scheduled appointments were actively followed up in the community. On each visit during the treatment and follow-up phase, a medical history was obtained, vital signs were checked, axillary temperature was measured, thick and thin blood smears were prepared from a fingerprick blood sample, and adverse events were documented. Venipunctures were performed on days 0, 7, and 28 to monitor hemoglobin levels, hematocrit, and leukocyte counts (differential and platelet) as well as liver damage and renal function parameters (serum alanine

Table 1. Baseline characteristics of pediatric outpatients treated with amodiaquine alone for uncomplicated *Plasmodium falciparum* malaria.

Characteristic	Value (n = 128)
Demography	
Female sex, no. (%)	69 (54)
Age, median (IQR), years	3.0 (1.8–5.1)
Weight, median (IQR), kg	12.6 (9.5–15.7)
Clinical parameters	
Duration of illness, median (IQR), days	2 (1–3)
Axially temperature, median (IQR), °C	37.6 (36.8–38.6)
Pulse, mean ± SD, bpm	134 ± 26
Parasitology	
Asexual *P. falciparum* density, median (range), parasites/μL	36,200 (1300–195,000)
Gametocyte carrier rate, proportion	1/128
Hematology	
Hemoglobin concentration, mean ± SD, g/dL	9.4 ± 1.4
Hematocrit, mean ± SD, %	28 ± 4
Leukocyte count, median (range), 10^3 cells/μL	8.3 (3.3–25.5)
Neutrophil count, median (range), 10^3 cells/μL	3.7 (0.3–18)
Platelet count, median (range), 10^3 cells/μL	130 (21–490)
Clinical chemistry	
Serum ALT level, median (range), IU/L	25 (10–660)
Serum bilirubin level, median (range), μmol/L	14 (3–65)
Serum creatinine level, mean ± SD, μmol/L	44 ± 11

NOTE. ALT, alanine aminotransferase; IQR, interquartile range; SD, standard deviation.

aminotransferase and serum creatinine concentrations, respectively). To distinguish recrudescences from new infections, aliquots of ethylenediaminetetraacetic acid–treated blood samples obtained on day 0 and on the day of reappearing asexual parasitemia were stored at −80°C for PCR-based genotyping analysis. In addition, blood samples were collected in Vacutainer tubes (BD) containing acid citrate dextrose and transport medium for adaptation of *P. falciparum* in culture on day 0 and the day of parasite reappearance for in vitro drug-sensitivity assays.

End points. To measure the prevalence of in vivo parasite resistance, we defined the primary end point as the PCR-corrected parasitological cure rate on day 28 ("adequate clinical and parasitological response" as defined by the WHO [21]). Cure was defined as initial clearance of asexual parasites by day 7 and subsequent sustained absence of microscopically detected asexual parasitemia through day 28. Secondary end points included the rate of recurrences (including reinfections) on day 28; fever and parasite clearance times; the difference in in vitro chemosensitivity profiles between parasite isolates obtained from patients with and those without subsequent asexual parasite recrudescence and between parasite isolates obtained before treatment with amodiaquine and at the time of asexual parasite recurrence; mean change in hemoglobin concentration from day 0 to 28; and the incidence of clinical and laboratory-determined adverse events.

Laboratory procedures. Dried thick and thin blood smears were stained with 20% Giemsa solution (pH 7.2). Parasite density was determined by enumerating the number of parasites per 200 white blood cells, assuming a total white blood cell count of 8000 cells/μL. For safety evaluation, differential blood counts were measured using with a semiautomated analyzer (AcT 5 Diff CP; Beckman Coulter), and clinical chemical analysis was done using a wet chemistry analyzer (Selectra E; Vitalab). To distinguish recrudescence from new infection, matched pairs of parasite isolates obtained on study admission and on the day of reappearing asexual parasitemia were compared using PCR-based genotyping analysis of repeat length polymorphisms in the *pfmsp2* gene locus [22]. We classified a recurrent asexual parasite infection after initial clearance as reinfection if the sizes of all electrophoretically separated PCR product bands detected for the day of reappearing asexual parasitemia were distinct from those detected for the day of study admission in at least 2 independent experiments.

Parasite adaptation and chemosensitivity testing. One milliliter of patient venous blood was collected and added to 4 mL of transport medium (Roswell Park Memorial Institute [RPMI] 1640 containing 10% albumin). Parasite suspensions were washed with normal RPMI 1640 and then cultured according to standard protocols (http://www.mr4.org). Parasite growth was monitored by light microscopy until a per-cycle increase in parasite

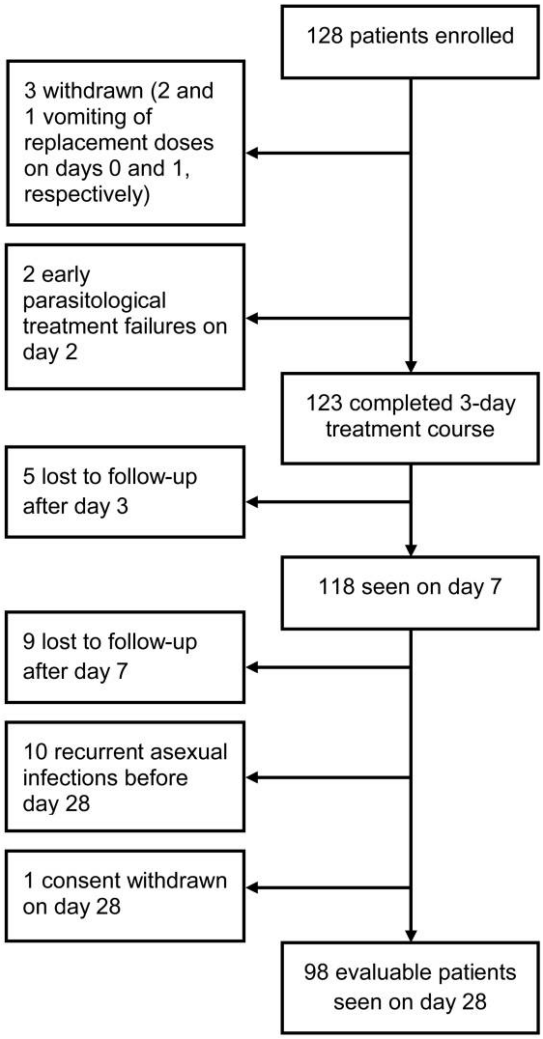

Figure 1. Study profile.

load of >2-fold was observed, at which point the culture was diluted 2-fold. An isolate was considered to be adapted at a stable 2-fold per-cycle growth rate. The chemosensitivity of culture-adapted parasites to amodiaquine dihydrochloride dihydrate (AQ; Sigma), to the principal active circulating human metabolite DEAQ (LGC), and to chloroquine diphosphate (CQ; Sigma) was determined by the radioisotope-incorporation method in duplicate experiments [23]. Briefly, the culture was diluted to 0.5% parasitemia and a final hematocrit of 1.5%. Two hundred microliters of blood-medium mixture was added to 96-well microtiter plates containing the drugs in duplicate 3-fold serial dilutions (CQ, AQ, and DEAQ in medium ranging from 540 to 0.75 nmol/L for AQ, from 580 to 0.8 ng/mL for DEAQ, and from 450 to 0.6 nmol/L for CQ) prepared from stock solutions in 70% ethanol (for CQ and AQ) and 90% methanol plus 10% HCl (for DEAQ). These were incubated at 37°C in a gas mixture of 90% N_2, 5% O_2, and 5% CO_2 for 66 h. Growth was measured by determining the incorporation of [^{3}H]-hypoxanthine, which was added during the last 18 h of incubation. Cells were lysed by freeze-thaw cycling of the plates. Cultures were harvested on fiberglass paper (PerkinElmer). Dried papers were immersed in scintillation fluid (PerkinElmer) before the amount of ionizing radiation was determined (1450 Microbeta Counter; Wallac). Results were expressed as the drug concentration required for 50% inhibition of [^{3}H]-hypoxanthine incorporation into parasite nucleic acid compared with drug-free control (50% inhibitory concentration [IC_{50}]), using regression analysis of the dose–response curve.

Data management and statistical analysis. Data were captured using specifically designed source documents and were subsequently double-entered into an electronic database (FileMaker Pro; version 5; FileMaker). A commercial software package (Stata/MP; version 10; StataCorp) was used for data analysis. Recrudescence-free estimates were calculated as Kaplan-Meier product limits. Patients with genotypically confirmed new infections and patients who withdrew or were lost to follow-up were right censored. Times to asexual parasite and fever clearance were calculated from the start of treatment to the first of 2 consecutive parasite-free blood smears and axillary temperature measurements <37.5°C, respectively, and were derived from Kaplan-Meier analyses. Mean drug concentrations at 50% inhibition of growth were calculated by fitting a polynomial regression model. Regression models with R^2 values <0.9 were not included in analyses (Microsoft Excel 2008).

RESULTS

Study cohort. A total of 128 patients were enrolled into the present study and received at least 1 dose of amodiaquine. The baseline demographic and clinical characteristics of the patients enrolled in this study are summarized in table 1. Seventy-three percent (94/128) of the patients were <5 year old, and 93% (119/128) had been ill for ≤3 days. Baseline hematological and clinical chemistry parameters were within the reference range for the community with the exception of platelet counts, which were below the lower limit of the reference range in 57% (72/126) of patients. Figure 1 details the flow of patients through the study. A total of 110 patients reached a primary efficacy end point.

In vivo response. Results for the clinical and parasitological responses to treatment with amodiaquine are summarized in

Table 2. Kaplan-Meier estimates of the in vivo responses to amodiaquine in pediatric outpatients with uncomplicated malaria.

Treatment response	Value
Mean time to fever clearance, h	28 (26–30)
Mean time to asexual parasite clearance, h	67 (65–69)
Estimate for no recurrent infection on day 28, %	72 (62–79)
Estimate for recrudescent infection on day 28,[a] %	82 (74–88)
Estimate for reinfection on day 28, %	17 (11–26)

NOTE. Data in parentheses are 95% confidence intervals.

[a] Cure rate.

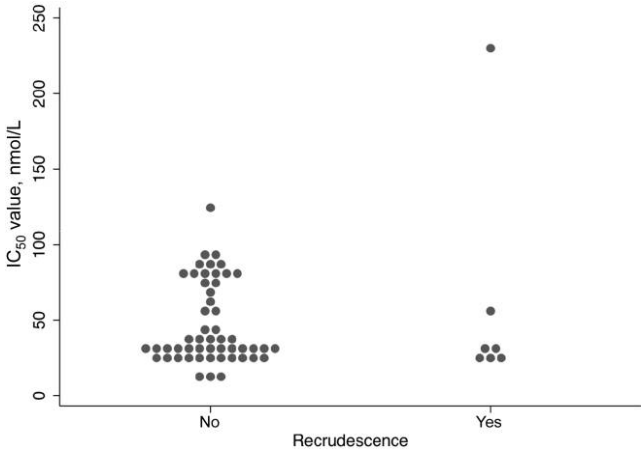

Figure 2. Comparison of 50% inhibitory concentration (IC_{50}) values for the response to desethylamodiaquine of isolates obtained before treatment from pediatric outpatients with and those without subsequent recrudescent infections.

table 2. Two parasitologically defined failures occurred on day 2 (asexual parasite density higher than baseline). All remaining patients were free of parasites by day 7. Between days 7 and 28, we detected the recurrence of asexual parasite infection in 29 patients. Genotyping analysis revealed that 12 recurrent infections were reinfections, 10 were recrudescences, and 5 were mixed (recurrent isolates containing persistent and new alleles), which were classified as recrudescences for the primary endpoint analysis. Two cases for which PCR results were indeterminate were classified as recrudescences. Day 28 estimates are given in table 2.

In vitro response. We first established the activity of DEAQ and AQ against reference laboratory strains V1/S [24] and 3D7 as resistant and sensitive controls, respectively. The AQ and DEAQ IC_{50} values for V1/S were 15 and 97 nmol/L, respectively, and the AQ and DEAQ IC_{50} values for 3D7 were 8 and 25 nmol/L, respectively, in agreement with the findings of previous studies using the same laboratory strains.

A total of 66 isolates (66/128 [46%]) obtained before treatment were tested for sensitivity to AQ and DEAQ, and a total of 19 isolates (19/29 [67%]) obtained from those with recurrent infections were tested. Of the 66 tested baseline isolates, 2 (3%) were resistant to amodiaquine but 17 (26%) were resistant to DEAQ when previously proposed arbitrary cutoffs of IC_{50} values of >30 nmol/L [6] and >60 nmol/L [25], respectively, were applied. The median AQ IC_{50} and DEAQ IC_{50} values for isolates obtained before treatment from patients with versus those without subsequent asexual parasite recrudescence were similar ($P > .3$ for both comparisons): 16 nmol/L (95% CI, 7–46 nmol/L) and 29 nmol/L (95% CI, 23–170 nmol/L) versus 16 nmol/L (95% CI, 13–19 nmol/L) and 34 nmol/L (95% CI, 30–46 nmol/L), respectively (DEAQ IC_{50} results are shown in figure 2). The median AQ IC_{50} and DEAQ IC_{50} values for isolates obtained at baseline versus at asexual parasite recurrence were also similar ($P > .5$, for both comparisons): 16 nmol/L (95% CI, 13–19 nmol/L) and 33 nmol/L (95% CI, 30–43 nmol/L) versus 13 nmol/L (95% CI, 9–20 nmol/L) and 41 nmol/L (95% CI, 27–71 nmol/L), respectively (DEAQ IC_{50} results are shown in figure 3). AQ and DEAC IC_{50} values for baseline isolates were highly correlated (Spearman's $\rho = 0.75$; $P < .001$) (figure 4). Similarly, AQ IC_{50} values for baseline isolates were also highly correlated with CQ IC_{50} values (Spearman's $\rho = 0.53$; $P < .001$).

Safety and tolerability. No serious adverse events were reported. A total of 74 clinical adverse events were observed after treatment. Adverse events commonly involved the gastrointestinal tract, the respiratory system, and the skin. Sixty-five (88%) of the adverse events were judged to be of mild intensity and 9 (12%) of moderate intensity, but none was judged to likely be related to amodiaquine intake. Most of the adverse events were also symptoms of malaria and, therefore, were likely to be related to disease. Changes in laboratory values after treatment were consistent with acute malaria and its resolution and are summarized in table 3. Absolute neutrophil count decreased from day 0 to day 7 and then further dropped by day 28. A decline to National Institute of Allergy and Infectious Diseases–defined grade 1 neutropenia (white blood cell count of <1200 cells/μL) [26] after treatment was observed in 3 (3%) of 96 patients.

DISCUSSION

P. falciparum resistance to commonly used antimalarial drugs is a dynamic and important problem for public health, one that requires frequent and systematic monitoring of the efficacy of

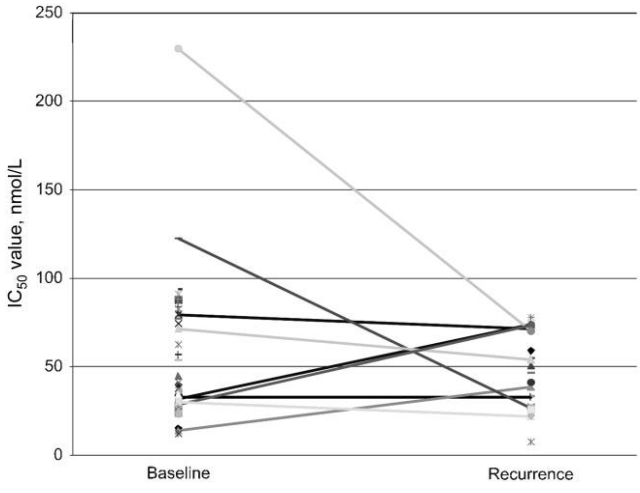

Figure 3. Comparison of 50% inhibitory concentration (IC_{50}) values for the response to desethylamodiaquine of isolates obtained from pediatric outpatients before treatment and at the time of asexual parasite recurrence. Paired samples are connected by straight lines.

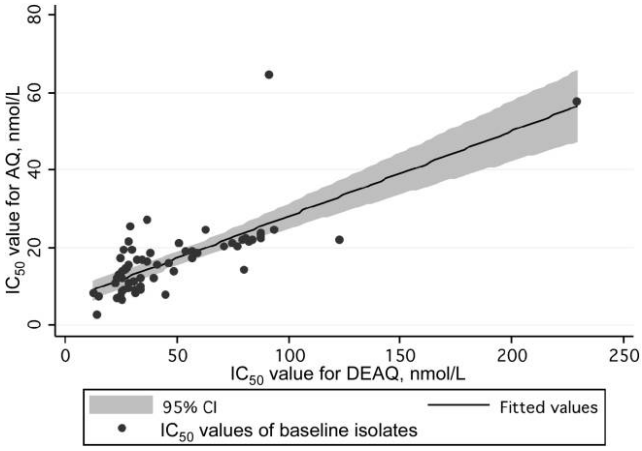

Figure 4. Scatter plot of 50% inhibitory concentration (IC_{50}) values for amodiaquine (AQ) and desethylamodiaquine (DEAQ) among *Plasmodium falciparum* isolates obtained from pediatric outpatients before treatment with amodiaquine. Values were fitted by linear prediction.

drugs in vivo and/or testing parasite sensitivity in vitro. Amodiaquine is one of the most important drugs for malaria control in Africa, presently being used in combination with artesunate as first-line treatment or alone as second-line treatment in 17 African countries [7].

Parasite resistance to amodiaquine and its major active metabolite, DEAQ, remains a concern. Various levels of in vivo resistance to amodiaquine have been reported in East Africa, with day 14 failure rates ranging from an alarming 42% in Muheza, Tanzania [12], to <6% across national sentinel sites in Uganda [10]. In our study, the initial clinical and parasitological response rates to treatment with amodiaquine were satisfactory—with the notable exception of 2 parasitologically defined early treatment failures occurring on day 2—but we observed a high rate (18%) of late recrudescences on day 28 (Kaplan-Meier estimate). This rate is >3 times higher than the recommended 5% cutoff for an efficacious antimalarial drug [19] and, thus, cautions against the continued use of amodiaquine as a cheap and widely available second-line drug. The use of a single genetic marker to distinguish between recrudescence and reinfection in the present study can be considered a minor limitation, one that may have resulted in a slightly inflated estimate of the failure rate.

On oral administration, amodiaquine is rapidly and extensively converted during the first pass in the liver to DEAQ, the main pharmacologically active metabolite [27–29]. Although amodiaquine has higher intrinsic in vitro activity against chloroquine-sensitive isolates than DEAQ [30], the in vivo efficacy of amodiaquine is almost entirely due to DEAQ [31–33]. We detected parasite resistance to amodiaquine in only 2 (3%) of the 66 tested isolates, whereas 17 (26%) of the isolates were resistant to DEAQ when previously proposed arbitrary cutoffs of IC_{50} values of >30 nmol/L [6] and >60 nmol/L [25], respectively, were applied. Our findings are in agreement with previous observations of near 100% prevalence of amodiaquine sensitivity among *P. falciparum* isolates obtained from African patients [34, 35], despite comparatively high rates of in vivo treatment failures [14, 15] and of in vitro resistance to DEAQ [15, 36]. This conflicting pattern could be explained by the rapid conversion of amodiaquine to DEAQ [32], resulting in minimal selective pressure on amodiaquine. The 2 early treatment failures in our study can therefore be interpreted as a result of (1) limited exposure to the highly active but quickly eliminated prodrug amodiaquine and (2) substantial parasite resistance to the main antimalarial metabolite of amodiaquine, DEAQ. On the other hand, the observed high correlation between in vitro responses to AQ and to DEAQ suggest the existence of cross-resistance between amodiaquine and DEAQ—and, indeed, of parasite resistance to amodiaquine. The clinical relevance of the latter interpretation remains elusive (in the absence of interest in intravenous amodiaquine treatment [37]), but parasite resistance to the parent drug may contribute to the decreasing in vivo response rates seen in patients treated with an oral course of amodiaquine alone or in combination [38].

Cutoff values have been proposed that would categorize in vitro responses of *P. falciparum* isolates as either sensitive or

Table 3. Changes in laboratory parameters among pediatric outpatients treated with amodiaquine for uncomplicated *Plasmodium falciparum* malaria.

	Day 0 to 7			Day 0 to 28		
Laboratory parameter	No.	Change	*P*	No.	Change	*P*
Hemoglobin concentration, mean, g/dL	116	−0.4 (−0.6 to −0.2)	.03	104	0.8 (0.5 to 1.1)	<.001
Leukocyte count, median, 10^3 cells/μL	116	1.1 (0.6 to 1.6)	<.01	104	0.4 (−0.6 to 1.1)	NS
Absolute neutrophil count, median, 10^3 cells/μL	104	−0.7 (−1.1 to −0.5)	<.001	92	−1.3 (−1.7 to −0.7)	<.001
Platelet count, mean, 10^3 cells/μL	116	210 (180 to 240)	<.001	103	180 (150 to 210)	<.001
Serum ALT level, median, IU/L	113	−2 (−5 to −1)	<.01	101	−2 (−4 to 0)	NS
Serum bilirubin level, median, μmol/L	111	−9 (−11 to −7)	<.001	98	−10 (−12 to −8)	<.001
Serum creatinine level, mean, μmol/L	113	0.4 (−2 to 3)	NS	101	−2 (−5 to 0.1)	NS

NOTE. Data in parentheses are 95% confidence intervals. ALT, alanine aminotransferase; NS, not significant.

resistant to inhibitors of parasite growth [39] on the basis of the observation that baseline in vitro phenotypes are correlated with the outcome of antimalarial treatment (i.e., the likelihood of a treated asexual blood stage infection to recrudesce). However, this relationship, especially for intermediate in vitro inhibitory phenotypes, is nonlinear and is likely confounded by other key determinants of treatment outcome (e.g., immune status of the patient, baseline parasite biomass, and pharmacokinetic variables) [40, 41]. In the present study, a relatively high proportion of baseline isolates (29%) had DEAQ IC_{50} values above the proposed cutoff of 60 nmol/L [25]; nonetheless, we failed to detect an association between baseline in vitro responses and subsequent risk of recrudescence.

In this study, we did not detect phenotypic signatures of selection for parasite resistance to amodiaquine or its major active metabolite, DEAQ, in field isolates after a single oral treatment course (43 nmol/L at recurrence vs. 33 nmol/L at baseline; $P = .6$). This may be due to the small number of isolates obtained from patients with asexual parasite recurrence. Whether selection for DEAQ-resistant parasites is amplified by repeated exposure of incompletely eliminated blood stage infections can be investigated only in longitudinal studies of the effects of repeated treatment with the same drug for sequential malaria episodes [42]. We did not determine the IC_{50} value for dihydroartemisinin (DHA). Such information may be valuable for examining the pattern of cross-resistance between DEAQ and DHA [43] and, thus, predicting the therapeutic life span of amodiaquine in combination with artesunate.

No serious adverse events were reported in our study. We observed an unexpected further drop in the median absolute neutrophil count after day 7. This contrasts with the typical pattern observed after antimalarial treatment [44, 45], in which an initial drop in absolute neutrophil count is followed by a subsequent recovery to normal values. In view of previous findings of potential bone marrow toxicity of amodiaquine when used for antimalarial chemotherapy [14, 46]—possibly mediated by bioactivation of amodiaquine to chemically reactive and cytotoxic intermediates [47] and/or induction of antidrug antibodies [48]—our results reemphasize the need for long-term safety monitoring in countries in which amodiaquine-artesunate is used as first- or second-line treatment.

In summary, our findings of an 18% failure rate in vivo and of a 29% rate of resistant responses to DEAQ in vitro indicates the presence of a considerable level of amodiaquine resistance in Kilifi District, on the Kenyan coast. This result warrants continuous attention to the value of amodiaquine combinations as first- or second-line treatment in areas with chloroquine-resistant parasites.

Acknowledgments

We are particularly indebted to the participating children and their parents. We thank the Pingilikani Dispensary Health Committee for facilitating the study and the Kenya Medical Research Institute Center for Geographic Medicine Research–Coast Clinical Trials Team for its role in patient recruitment. Special thanks go to nurses Ann Kithinji, Joy Lewa, and Crispinah Kaulu for providing nursing care and study drug administration.

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PATIENT NUMBER: _____

APPENDIX II

INFORMED CONSENT STATEMENT AND PATIENT INFORMATION SHEET

Title: **A clinical audit of amodiaquine in relation to the potential value of new amodiaquine analogues in the treatment of paediatric outpatients with uncomplicated *Plasmodium falciparum* malaria**

Investigator: **Dr. Steffen Borrmann**
KEMRI/Wellcome Trust Research Programme
On the grounds of Kilifi District Hospital
P.O. Box 230, Kilifi - 80108

Sponsor: **University of Liverpool, United Kingdom**
Medicines for Malaria Venture, Switzerland

Who is carrying out this research?
We invite you and your child to take part in a research study being conducted by Kenya Medical Research Institute (KEMRI), a national organisation with the government mandate to learn more about the treatment and prevention of illnesses in Kenya. The participation is entirely voluntary.
The KEMRI unit in Kilifi has worked mutually with Kilifi District Hospital for the last 13 years. Since two years, KEMRI has started to work closely with the community and the Dispensary Health Committee in Pingilikani, the Kilifi District Medical Officer of Health (Ministry of Health) and the Provincial Medical Officer in order to gain knowledge that will facilitate the development of improved health services for all Kenyans.
We wish to learn more about the value of amodiaquine as the current first-line treatment for patients with mild malaria at Pingilikani dispensary. This study will also help to assess the potential usefulness of new drugs that are similar to amodiaquine.

What is the purpose of the study?
We are trying to learn more about the best way to treat malaria in Kenya. Coartem© is being introduced as the first-line drug for the treatment of cases with mild malaria in Kenya but it is not yet available in the public health care system. In the meantime, amodiaquine is used as a temporary replacement for the failing chloroquine and sulfadoxine-pyrimethamine (S/P). Our aim is to collect important information on the current clinical value of amodiaquine in the treatment of childhood malaria.

What are the study procedures?
Today:
1) A study doctor will examine your child today.
2) A blood sample (volume equivalent to a teaspoon) will be collected from a forearm vein of your child to examine for malaria parasites and indicators of your child's health (haematology and clinical chemistry). In addition, a small share of this blood sample will be stored on filter paper for laboratory tests that will not impact on the health care of your child. In addition, blood will also be used to study the malaria parasites in a test tube and the presence of factors potentially reducing the tolerance of the body to amodiaquine. Some of these samples will be stored and later shipped to overseas laboratories for analyses requiring technology that is not yet available in Kenya.
3) Your child will then receive the first dose of amodiaquine under supervision by a member of the KEMRI staff.

Continuation of treatment over the next 2 days:
1) Treatment will continue once daily over the next 2 days (tomorrow and the day after tomorrow). We ask you to bring your child once daily to our clinic for dosing (tomorrow and the day after tomorrow). Alternatively, your child can be admitted

to our observation ward for 3 days to allow the administration of amodiaquine at the exact day times.

2) Before administration of treatment your child will be examined and then pricked in the finger once every day to check for malaria parasites.

Follow-up over 4 weeks:

1) You will be asked to bring your child to the clinic three times over the next 4 weeks so that the success of the treatment can be judged (in 3 days and after 1 and 4 weeks). At each of the follow-up visits, your child will be examined by the study doctors and, a small amount of blood will taken most of the times by finger prick to examine for malaria parasites. In case of missing an appointment, a study team member will visit your child at your home to either assess your child at home or bring your child to the clinic for assessment.

Additional procedures:

2) *Blood samples*: To monitor potential side effects of the study medicines we will need to to take samples on two more occasions (volume equivalent to a half a teaspoon taken from a forearm vein). These samples will be taken after 1 and 4 weeks. In some patients these samples will also be used to study malaria parasites in a test tube and to store a small share on filter paper for laboratory tests that will not impact on the health care of your child. Some of these samples will be stored and later shipped to overseas laboratories for analyses requiring technology that is not available in Kenya.

If, at any time, amodiaquine does not seem to be working well, your child will be given another malaria treatment (Coartem©).

For the 4 weeks that your child is participating in the study, you will have access to the clinic at all times, day and night. If your child becomes unwell you are encouraged to bring your child to the clinic without delay.

The study doctors may withdraw your child from the study for the following reasons:

1) If your child receives malaria medicines not prescribed by the study doctors;
2) If your child has a serious adverse event or needs to be hospitalised;
3) If we are unable to locate your child within 6 hours during the first three days or within 24 hours during the follow-up period.

Are there any risks associated with the study?

1) Side effects following treatment with the study medications could occur. Generally, side effects (such as nausea, headache, dizziness) are expected to be mild and only short-lived.
   Your child will be monitored closely after receiving treatment for malaria with the study medications for any possible side effects of the drugs and will receive appropriate medical care for any problem that happens during the course of the study.
2) Severe malaria: Your child may develop malaria that is severe even after receiving treatment with amodiaquine. If your child shows any evidence of severe malaria (including persistent vomiting, anaemia, convulsions, deep breathing, or coma) your child will be admitted to the paediatric ward at Kilifi District Hospital for appropriate treatment.
3) Blood collection: The risks of drawing blood by finger prick or venepuncture include temporary discomfort from the needle prick, bruising, skin infection, and fainting. The amount of blood removed will be too small to affect your child's health.

Are there any benefits associated with the study?

1) Your child will receive clinical care from a dedicated team of clinicians and nurses. This will include access to the clinic at all times, day and night, whenever

your child becomes unwell.

2) The knowledge gained from this study will help Kenya to determine the best treatment option for uncomplicated malaria.

Am I entitled to compensation?
After enrolment in the study, you will not be charged for clinic visits or treatment during the next 12 weeks. We will reimburse any transport costs incurred for clinic visits and meals will be provided when your child is admitted for observation and treatment administration.
If your child becomes ill or is injured as a result of participation in this clinical study, medical treatment will be provided and the sponsor will pay the reasonable cost of such treatment.

Can I ask questions?
You have the right to ask the doctor any questions concerning this study at any time. You will be told if any new information, which may affect your child's health, becomes available during the study.

Are there alternatives to participation; is participation voluntary?
Your child's participation in this study is completely voluntary. If you decide that you do not want to participate in the study or decide to withdraw your child from the study at any time and for any reason, this will not affect your child's care at the Pingilikani dispensary. During the participation of your child in the study, you will be informed promptly of any new information that may influence your willingness to continue participation in the study.

Are there any consequences of withdrawal?
Should you or your study doctors decide to withdraw your child from the study before your child has finished the course of amodiaquine, then your child will receive the local standard treatment for malaria from the study team, which takes three days. If the child is withdrawn from the study after completion of the course of study medicines, the study team will provide then no further care.

How are the study results used?
The findings from this study may be reported in a medical journal. The study participants will not be identified by name. After the study is completed, you may request an explanation of the study results.
The medically important results from the blood examinations will routinely be written in your child's health care booklet at the next visit after the blood sample was obtained.

Storage of samples
For practical and technical reasons, often more blood needs to be taken than absolutely necessary for a particular test. For instance, a repeat test may be required in case of an abnormal test result. Rather than discarding the remaining sample as it is normally done, we would like to store it. This will enable us to possibly use some samples in the future to help finding solutions for problems of importance to the public health in Kenya.
We will only use stored samples for new studies if the National Ethical Review Committee will grant us permission. Because the test results from possible future studies will have no implication for the current illness of your child, we will not normally inform you about these results unless you wish to be informed whenever future tests are done.

PATIENT NUMBER: _____

What if I have questions about the study or my child's rights as a research subject?

If you/your child have any questions about this study, if there are things that you/your child do not understand, or if your child has a study-related injury, please contact:
Drs S. Borrmann or P. Sasi, KEMRI/Wellcome Trust Research Programme
On the grounds of Kilifi District Hospital, P.O. Box 230, Kilifi - 80108, (Tel: 041-522063).
If you have any questions about your child's rights as a research study participant, please contact:
Dorcas Kamuya, KEMRI Community Liaison Officer
On the grounds of Kilifi District Hospital, P.O. Box 230, Kilifi - 80108, (Tel: 041-522063).

You will be given a signed copy of this form.

PATIENT NUMBER: _____

INFORMED CONSENT STATEMENT AND PATIENT INFORMATION SHEET

Title: **A clinical audit of amodiaquine in relation to the potential value of new amodiaquine analogues in the treatment of paediatric outpatients with uncomplicated *Plasmodium falciparum* malaria**
Investigator: **Dr. Steffen Borrmann**
Sponsor: **University of Liverpool, United Kingdom**

I _____ being mother/father/legal guardian or representative of

_____ (name of child) declare that I have understood the objectives and purposes of this study. I agree that my child may participate in this study. I am aware that I can withdraw my child from the study at any time without any consequence to my child or to me.

Name of parent/legal representative

Signature or Thumbprint * of parent/ legal representative Date/Time

*If the parent or guardian is unable to read and/or write, an impartial witness should be present during the informed consent discussion. After the written informed consent form is read and explained to the parent or guardian, and after they have orally consented to their child's participation in the trial, and have either signed the consent form or provided their fingerprint, the witness should sign and personally date the consent form. By signing the consent form, the witness attests that the information in the consent form and any other written information was accurately explained to, and apparently understood by, the parent or guardian, and that informed consent was freely given by the parent or guardian.

Name of Person Witnessing Consent (printed)

Signature of Person Witnessing Consent Date/Time

Signature of Person Conducting Informed Consent Discussion Date/Time

INFORMED CONSENT STATEMENT AND PATIENT INFORMATION SHEET

Patient Information Sheet

TITLE: A randomised open label study to assess the safety and efficacy of dihydroartemisinin-piperaquine (Artekin™) compared with lumefantrine-artemether (Coartem®) for the treatment of uncomplicated *Plasmodium falciparum* malaria in Kenyan children.

Protocol No**:** **ST3073+ST3074 DM040010**
Sponsor**:** **Sigma-Tau**
Financial support: **Medicine for Malaria Venture (MMV)**
Investigator**:** **Dr. Steffen Borrmann**

Who is carrying out this research?

We invite you and your child to take part in a research study being conducted by Kenya Medical Research Institute (KEMRI), which is a national organisation with a government mandate to learn more about the treatment and prevention of illnesses in Kenya. ***Taking part is entirely voluntary.***

The KEMRI unit in Kilifi has worked mutually with Kilifi District Hospital for the last 13 years. Since two years, KEMRI has started to work closely with the community and the Dispensary Health Committee in Pingilikani, the Kilifi District Medical Officer of Health (Ministry of Health) and the Provincial Medical Officer to improve the medical and laboratory facilities at Pingilikani Health Centre. The aim is to learn more about treatment of illnesses and to gain knowledge that will facilitate the development of improved services available for both prevention and treatment of diseases of all Kenyans.
We are currently trying to learn about a new ***experimental*** antimalarial drug called Artekin™. Artekin™ is manufactured by the sponsor of this study (Sigma-Tau, Industrie Farmaceutiche Riunite S.p.A., Rome, Italy). KEMRI is carrying out this study in collaboration with Medicine for Malaria Venture.

What is the purpose of the study?
We are trying to learn more about the best way to treat malaria in Kenya. Currently, Coartem© is being introduced as the first-line drug for the treatment of cases with mild malaria in Kenya. However, Coartem© requires to take 6 doses at exact time points over 3 days. Artekin™ is a new alternative that is already successfully used in some countries in Southeast Asia and requires only 3 doses once daily over 3 days. We are trying to find out whether Artekin™ can cure malaria at least as effectively and safely as Coartem©. Five hundred children with mild malaria will participate in this study. We are requesting your permission for your child to participate in the study if he/she has malaria.

How the study is done?

If you agree, the child under your care will be treated with one of the above study medicines for his/her present malaria illness. After the treatment, your child will be followed for 12 weeks to see if the malaria infection is completely cured. ***It will be your responsibility to bring your child to the clinic for follow up visits.*** If your child is not completely cured by the study medicines, he/she will then be given treatment according to the standard practice in Kenya. A process of randomisation will determine the study medicine that your child will receive. Randomization means that your child will receive one of the two medicines by chance. You are being asked to allow your child (child under your care in the case of a legal guardian), to participate in this study for up to 12 weeks or until such time as you or the study doctors decide that your child should no longer participate in the study. The study doctors may withdraw your child from the study for the following reasons:

1) If your child receives malaria medicines not prescribed by the study doctors
2) If your child needs to be hospitalised;
3) If we are unable to locate your child within 6 hours during the first three days or within 24 hours during the follow-up period.

The sponsor may discontinue the study at any time, and for any reason.

What are the study procedures?

Today:

1) The study doctors will examine your child today.
2) A blood sample (volume equivalent to a teaspoon) will be collected from a forearm vein of your child to examine for malaria parasites and indicators of your child's health (haematology and clinical chemistry). In addition, a small share of this blood sample will be stored on filter paper for future laboratory tests that will not impact on the health care of your child. In some patients blood will also be used to assess to study the effect of the study medicines against the malaria parasites in a test tube.
3) In addition, the heart of your child will be examined by recording its function with electrodes placed on the chest of your child (electrocardiogram).
4) If the diagnosis of malaria is confirmed, and your child is eligible for the study, treatment with Artekin™ or Coartem© will be started.

Treatment over 3 days:

1) Treatment will be given over 3 days (today, tomorrow and the day after tomorrow). If your child receives Coartem© he/she will be admitted to our observation ward for 3 days to allow the administration of Coartem© at the exact day and night times. If your child receives Artekin™ you will be asked to bring your child to Pingilikani Health Centre tomorrow and the day after tomorrow always at the same time of the day.
2) Before administration of treatment your child will be examined and then pricked in the finger once every day to check for malaria parasites.
3) On the third day the heart of your child will again be examined by recording its function using harmless and small electrodes placed on the chest of your child (electrocardiogram).

Follow-up over 12 weeks:

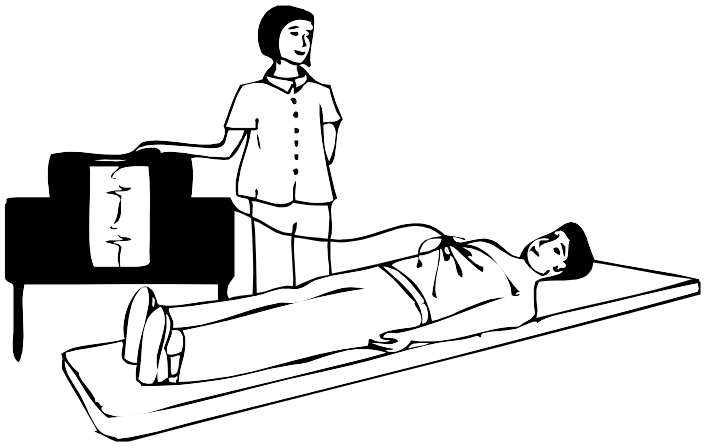
1) You will be asked to return to the clinic at least 11 more times over the next three months so that the success of the treatment can be judged. At each of the

follow-up visits, your child will be examined by the study doctors and, a small amount of blood will taken most of the times by fingerprick to examine for malaria parasites. In case of missing an appointment, a study team member will visit your child at your home to either assess your child at home or bring your child to the clinic for assessment.

Additional procedures:

2) *Blood samples*: To monitor potential side effects of the study medicines we will need to examine the child's blood regularly, and for this type of examination a samples of 2 mL (volume equivalent to a teaspoon) are needed from a forearm vein. Blood samples will be taken on the day after the end of the treatment (day 3), and then again after 1, 4, 6, 8 and 12 weeks. In some patients blood will also be used to study the effect of the study medicines against the malaria parasites in a test tube.
3) *Heart check*: 4 weeks after the start of the treatment the heart of your child will be examined by recording its function using harmless and small electrodes placed on the chest of your child (electrocardiogram).

Illustration of how an electrocardiogram is performed.



If, at any time, the study medicine does not seem to be working well, your child will be given a standard malaria treatment.

For the 12 weeks that your child is participating in the study, you will have access to the clinic at all times, day and night. If your child becomes unwell you are encouraged to bring your child to the clinic without delay.

Are there any risks associated with the study?

1) Side effects following treatment with the study medications could occur. Generally, side effects (such as nausea, headache, dizziness) are expected to be mild and only short-lived.

   Your child will be monitored closely after receiving treatment for malaria with the study medications for any possible side effects of the drugs and will receive appropriate medical care for any problem that happens during the course of the study.
2) Randomization: Your child will be allocated one of the two study medicines by chance. The treatment your child receives may prove to be less effective or

to have more side effects than the other study treatment or than other available treatments. This will not be known until after the study is completed.

3) Severe malaria: Your child may develop malaria that is severe even after receiving treatment with study medications. If your child shows any evidence of severe malaria (including persistent vomiting, anaemia, convulsions, deep breathing, or coma) your child will be admitted to the Paediatric Ward of Kilifi District Hospital for appropriate treatment.
4) Blood collection: The risks of drawing blood by fingerprick or venepuncture include temporary discomfort from the needle prick, bruising, skin infection, and fainting. The amount of blood removed will be too small to affect your child's health.
5) Unknown Risks: The study medicines may have yet unknown side effects. The investigators will inform you if any new information becomes available.
6) Confidentiality: Participation in research may involve a loss of privacy, but ***everything possible will be done to keep the information about your child confidential, and will not be disclosed to any unauthorized persons.***
7) ***By signing this informed consent, you give permission that the monitors, auditors and regulatory authorities will be granted direct access to your child's medical records, for verification of the clinical trial procedures. The data will be provided without violating confidentiality of you or your child.***

Are there any benefits associated with the study?

1) Your child will receive clinical care from a dedicated team of clinicians and nurses. This will include access to the clinic at all times, day and night, whenever your child becomes unwell.
2) The knowledge gained from this study will help your country in determining the best treatment option for uncomplicated malaria.

Am I entitled to compensation and costs?

After enrolment in the study, you will not be charged for clinic visits or treatment during the next 12 weeks. We will reimburse any transport costs incurred for clinic visits and meals will be provided when your child is admitted for observation and treatment administration.

If your child becomes ill or is injured as a result of participation in this clinical study, medical treatment will be provided and the sponsors will pay the reasonable cost of such treatment and compensation.

Can I ask questions?
You have the right to ask the doctor any questions concerning this study at any time. You will be told if any new information on the medicine, which may affect your child's health, becomes available during the study.

Please notify your study doctor, Dr. Steffen Borrmann at telephone number - 041-522063, Kilifi District Hospital, immediately if your child experience ***a side effect, an injury, any symptom or complaint.***

Are there alternatives to participation, is participation voluntary?
Your child's participation in this study is completely voluntary. If you decide that you do not want to participate in the study or decide to withdraw your child from the study at any time and for any reason, this will not affect your child's care at the Pingilikani Health Centre, where a KEMRI team including a clinical officer specialized in child health provide care for medical problems. During the participation of your child in the study, you will be informed promptly of any new information that may influence your willingness to continue participation in the study.

Are there any consequences of withdrawal?

Should you or your study doctors decide to withdraw your child from the study before your child has finished the course of study medicines, then your child will receive the local standard treatment for malaria from the study team, but after the standard treatment has been given, medical care will no longer be provided by the study team. If the child is withdrawn from the study after completion of the course of study medicines, the study team will provide then no further care.

How are the study results used?
The findings from this study may be published in a medical journal. The study participants will not be identified by name. After the study is completed, you may request an explanation of the study results. At the end of the study the study results will be presented at a community meeting at Pingilikani Health Centre.
In addition, the results of this study will help to identify the best treatment for mild malaria in Kenya.

***People whom you may contact regarding the study*:**

Thank you for taking the time to read (or have read to you) the information about this research study. If you have any questions or concerns now or at any time, about the research study, your child's safety or your rights, please ask your doctor, his/her research study staff or the contact person(s) indicated below. If you enter this research study, you can contact the following people listed below if you have any questions or worries.

For Medical or study related questions, contact:

Dr. Steffen Borrmann will have the overall responsibility for conducting the study while Dr. Philip Sasi will be in charge of the day-to-day running of the study and the clinic. They are available at the hospital at 041-522063.

For any concerns regarding the ethics of the study, contact:
Ms. Kithinji , Tel: (254) (020-2722541, Secretary KEMRI Ethical Review Committee.

This study has been approved by the KEMRI Ethical Review Committee. If you have any questions or concerns about your participation in the study at a later date, please feel free to ask Dr. Steffen Borrmann or the Secretary of the Ethical Review Committee.

If you agree that your child may participate in the study, we invite you to sign the consent form below. A copy of this form will be given to you.

You will be given a signed copy of this form.

CONSENT FORM

CONSENT FORM FOR PARTICIPATION IN RESEARCH PROJECTS AND CLINICAL TRIALS

TITLE: A randomised open label study to assess the safety and efficacy of dihydroartemisinin-piperaquine (Artekin™) compared with lumefantrine-artemether (Coartem®) for the treatment of uncomplicated *Plasmodium falciparum* malaria in Kenyan children.

Principal Investigator: ***Steffen Borrmann***

Address: ***Kemri Wellcome Trust***
Kilifi Hospital
Kenya

Contact Number: ***041-522063***

I, …… mother/father/legal representative declare that I have understood the objectives and purposes of this study. I agree that my child may participate in this study.
I am aware that I can withdraw my child from the study at any time without any consequence to my child or to me.

Name of parent/legal representative

Signature or Thumbprint * of parent/ legal representative Date/Time

*If the parent or guardian is unable to read and/or write, an impartial witness should be present during the informed consent discussion. After the written informed

consent form is read and explained to the parent or guardian, and after they have orally consented to their child's participation in the trial, and have either signed the consent form or provided their fingerprint, the witness should sign and personally date the consent form. By signing the consent form, the witness attests that the information in the consent form and any other written information was accurately explained to, and apparently understood by, the parent or guardian, and that informed consent was freely given by the parent or guardian.

Name of Person Witnessing Consent (printed)

Signature of Person Witnessing Consent Date/Time