Journal of Infection and Public Health (2017) 10, 110—119

http://www.elsevier.com/locate/jiph

Synergism between Pfcrt and Pfmdr1 genes could account for the slow recovery of chloroquine sensitive *Plasmodium falciparum* strains in Ghana after chloroquine withdrawal

Kwame K. Asare[a,e,f], **Johnson N. Boampong**[a], **Nancy O. Duah**[b], **Richmond Afoakwah**[a], **Rakesh Sehgal**[c], **Neils B. Quashie**[d,b,*]

[a] *Department of Biomedical and Forensic Sciences, University of Cape Coast, Cape Coast, Ghana*
[b] *Epidemiology Department, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, PO Box LG581, Legon, Ghana*
[c] *Department of Medical Parasitology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India*
[d] *Center for Tropical Clinical Pharmacology and Therapeutics, School of Medicine and Dentistry, College of Health Sciences, University of Ghana, PO Box GP4236, Accra, Ghana*
[e] *Department of Protozoology, Institute of Tropical Medicine (NEKKEN), Nagasaki University Sakamoto 1-12-4, Nagasaki 852-8523, Japan*
[f] *Leading Program, Graduate School of Biomedical Sciences, Nagasaki University, Japan*

Received 3 December 2015; received in revised form 4 February 2016; accepted 20 February 2016

KEYWORDS
Malaria;
Drug resistance;
Haplotype;

Summary Unlike other countries, the chloroquine resistant marker Pfcrt T76 mutant has remained fairly stable in Ghana several years after official disuse of chloroquine. Certain mutations in Pfmdr1 may potentiate Pfcrt T76, offering a possible explanation for this observation. To understand the phenomenon, the co-existence of mutations in Pfmdr1 with Pfcrt T76 in Ghanaian *Plasmodium falciparum*

* Corresponding author at: Center for Tropical Clinical Pharmacology and Therapeutics, School of Medicine and Dentistry, College of Health Sciences, University of Ghana, PO Box GP4236, Accra, Ghana.
E-mail addresses: kwamsare@hotmail.com (K.K. Asare), jonboamus@yahoo.com (J.N. Boampong), nduah@noguchi.ug.edu.gh (N.O. Duah), aforich2007@yahoo.com (R. Afoakwah), sehgalpgi@gmail.com (R. Sehgal), nquashie@noguchi.ug.edu.gh (N.B. Quashie).

http://dx.doi.org/10.1016/j.jiph.2016.02.004

Recovery;
Synergism;
Gene;
Plasmodium falciparum;
Chloroquine;
Amodiaquine;
Quinine;
Pfcrt;
Pfmdr1

isolates was studied. The reported presence of parasites with reduced sensitivity to amodiaquine and quinine in the country was also studied. Blood samples collected from confirmed malaria patients presenting at health facilities in two distinct ecological zones were analyzed. The prevalence of Pfcrt K76T and the five point mutations in Pfmdr1 were determined using nested PCR followed by RFLP analysis. The association between genes was determined by chi square analysis, and synergism between the two genes was ascertained using the Jonckheere—Terptra (J—T) test followed by Monte Carlo simulation (MCS). Nearly fifty-four percent (53.7%) of the *P. falciparum* isolates examined had the Pfcrt T76 gene, out of which 18.3% had both K76 and T76 alleles. Mutations at codon 86, 184, 1034, 1042 and 1246 of the Pfmdr1 gene were detected in 36.0%, 87.9%, 71.0%, 91.6% and 8.4% of the isolates, respectively. The haplotypes of Pfmdr1 present were NFCDD (43.46%), YFCDD (27.57%), NFSDD (7.48%), NYSNY (5.14%) and YFSDD (4.67%). Pfcrt T76 was significantly associated with a double mutation at codon 86 and 184 of Pfmdr1 (YF; $\chi^2 = 18.045$, $p = 0.006$). Associations were observed between Pfcrt K76T and Pfmdr1 triple mutation at codons 86, 184 and 1034 (NFC; $\chi^2 = 13.770$, $p = 0.032$ and YFC; $\chi^2 = 16.489$, $p = 0.011$). The J—T test showed significant synergism between Pfcrt 76 and Pfmdr1 polymorphisms ($p < 0.0001$), which was confirmed by MCS at 99% CI. Synergism between Pfcrt and Pfmdr1 mutant genes could account for the slow recovery of chloroquine sensitive *P. falciparum* in Ghana. The same phenomenon could explain resistance to amodiaquine and quinine. The outcomes of this study also indicated a possible emergence of artemether-lumefantrine resistance in Ghana.

Introduction

Several years after the replacement of chloroquine as a first line antimalarial drug in Malawi, Ethiopia and Tanzania, among others, chloroquine (CQ) sensitive *Plasmodium falciparum* has recovered [1—4]. The re-emergence of CQ-sensitive *P. falciparum* resulted from an increased dominance of wild-type parasites that have lysine at codon 76 of the Pfcrt gene [5—8]. Interestingly, the period of this recovery coincided with a reduction in point mutations of the Pfmdr1 genes, such as Asn86Tyr, Asn1042Asp and Asp1246Tyr [9]. This observation strongly suggests a possible link of Pfcrt with Pfmdr1 mutations in a modulation of the sensitivity of *P. falciparum* to CQ.

In our previous report, we showed the main CQ resistant marker K76T of the Pfcrt gene has remained fairly stable with a gradual decrease in certain parts of Ghana compared to Malawi [10,11]. Persistence of chloroquine resistant parasites in Ghana after the changes in anti-malarial drug policies has also been observed in other countries [12—14]. Though the prevalence of mutations in the Pfcrt gene on chromosome 7 and the Pfmdr1 gene on chromosome 5 has been determined individually in Ghana, the interplay between them has yet to be assessed and discussed in detail. Study is needed due to reports that indicate both Pfmdr1 gene duplication and mutations at codons 86, 184, 1034, 1042 and 1246 are associated with resistance to chloroquine, mefloquine, quinine and artemisinin derivatives [12,15,16]. Of particular interest is how different haplotypes of Pfmdr1 contribute to specific antimalarial drug resistance. Specifically, the combined effect of Y184F, N1042D and D1246Y (FDY) haplotype isolates from Africa, Asia and South America have been reported to be associated with CQ resistance phenotypes [17—19]. Additionally, study is needed for the S1034C, N1042D and D1246Y (CDY) isolates from South America that are associated with quinine resistance [20], as well as for the N86, F184 and D1246 (NFD) isolates from Tanzania and Mozambique that are associated with artemether lumefantrine recrudescence [21,22].

This study investigated the impact of the combined mutations in the Pfmdr1 and Pfcrt mutant genes on the delayed restoration of sensitivity of malarial parasites to CQ in Ghana. Additionally, the study sought an explanation for the reports of increasing levels of *P. falciparum* resistance to quinine and amodiaquine.

Methods

Study sites

This report describes a cross-sectional study conducted in Ghana health facilities located in areas

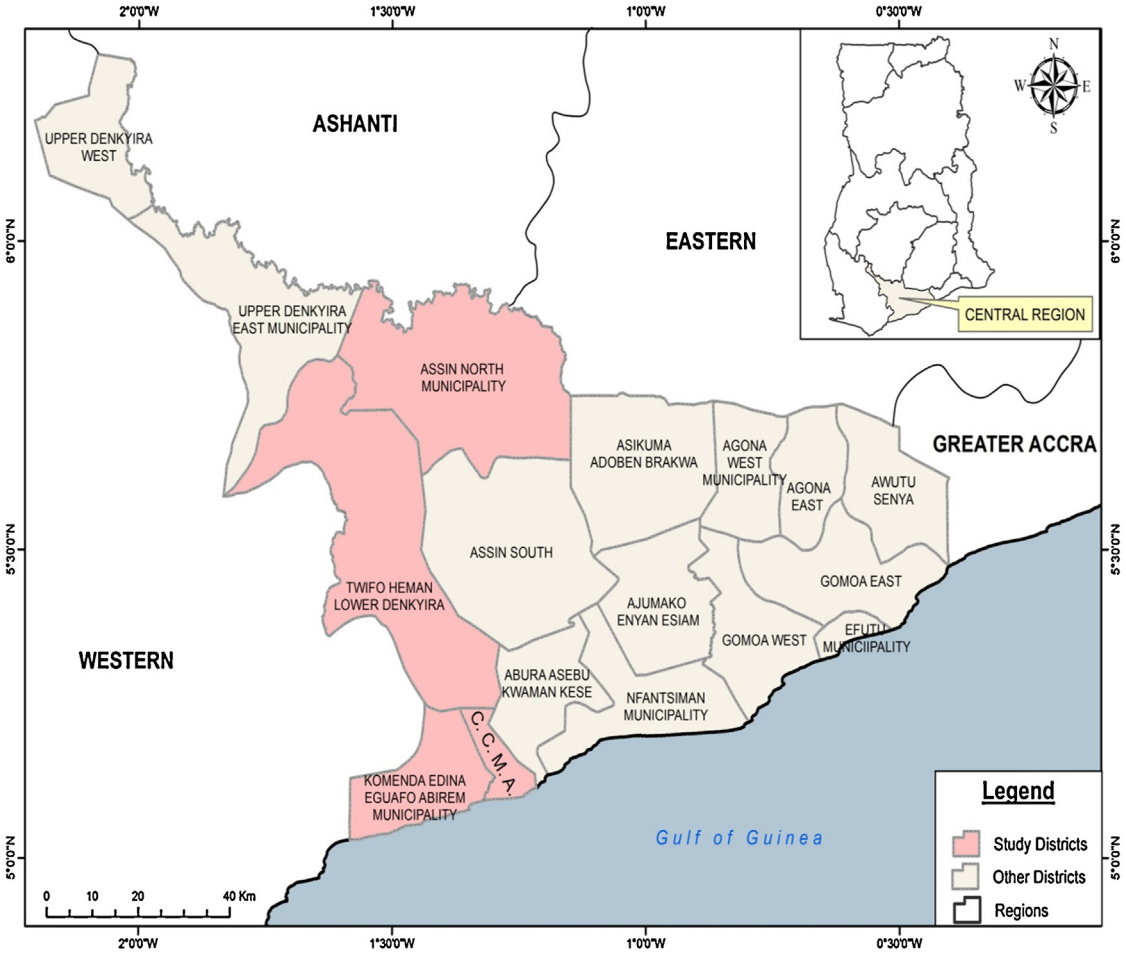

Figure 1 Map of the Central Region of Ghana. Map showing the district where the study was conducted (insert is the map of Ghana showing the position of the Central Region).

representing two distinct ecological zones, both forest and coastal. The facilities are the Twifo Praso district and St. Francis Xavier hospitals located in the forest zone, and the Cape Coast Metropolitan hospital and Elmina health center which are located in the coastal zone (Fig. 1). Apart from ecology, other considerations for selection of these sites were based on malaria endemicity, prevalence of Pfcrt 76T mutations and the level of chloroquine usage as determined in our previous study [10].

As in other areas in Ghana, treatment of febrile illness with chloroquine was the mainstay of malaria control until 2005 when field-based evidence indicated the presence of *P. falciparum* isolates resistant to the drug. Based on this evidence, and upon the recommendation of the WHO and other organizations, in 2005 Ghana officially changed from the use of chloroquine to an artemisinin-based combination therapy (ACT) as the first choice antimalarial drug for treatment of uncomplicated malaria. An artesunate—amodiaquine combination is the antimalarial drug most used in the selected study sites. The use of long lasting insecticide-treated nets (LLINs), indoor residual spraying (IRS) and intermittent preventive treatment among pregnant women (IPTp) remains the major malaria intervention measures in these areas.

Ethical considerations

All patients presenting with symptoms of malaria to the outpatient department of the selected health facility on the day of recruitment were screened for study inclusion. Only those with *P. falciparum* infection detected by microscopy were recruited to participate. The purpose of the study was explained to the adults. For children, both the children and their parents or guardian were encouraged to ask questions if clarity was needed. Patients were only recruited after giving consent.

Table 1 Prevalence of mutations in Pfmdr1 and Pfcrt genes in *Plasmodium falciparum* isolates.

	Polymorphisms (%)		
Gene/codon position	Wild-type	Mutant type	Mixed
Pfmdr1			
86	137 (64.0)	77 (36.0)	None
184	26 (12.1)	188 (87.9)	None
1034	62 (29.0)	152 (71.0)	None
1042	18 (8.4)	196 (91.6)	None
1246	179 (83.6)	18 (8.4)	17 (7.9)
Pfcrt			
76	60 (28.0)	115 (53.7)	39 (18.3)

Sample collection

A total of 618 patients were screened, out of which 217 were qualified to participate in the study. Basic biodata of all participants was collected and a 1 ml blood sample was collected and stored in tubes containing EDTA for parasitological analysis. A filter paper blood blot was prepared for each selected patient and later air-dried and stored at −20 °C in zip-locked plastic bags containing a silica gel for molecular analysis.

Malaria microscopy

Thick and thin blood films were prepared from the patient's blood samples and stained with 10% Giemsa. The slides were examined under oil immersion with the light microscope for the presence of *P. falciparum*.

Detection of Pfcrt and Pfmdr1 polymorphisms

Nested PCR for the amplification of Pfcrt and Pfmdr1 genes, followed by restriction fragment length polymorphism (PCR-RFLP), was used to detect mutations in the genes following published protocols [23,24]. Presence of mutations in Pfcrt at position 76 and Pfmdr1 at positions 86, 184, 1034, 1042 and 1246 were determined.

Data analysis

Data were organized using Microsoft Office Excel 2007 (Microsoft Corporation) and analyzed with SPSS Statistical Software version 16 (SPSS Inc.). Simple proportion was used to estimate the prevalence of the mutation. Statistical association between polymorphisms in Pfmdr1 and Pfcrt at codon 76 was determined with Pearson chi-square test. A p-value ≤ 0.05 was considered statistically significant. Additionally, synergism between Pfcrt and Pfmdr1 gene was assessed using the Jonckheere—Terpstra test and statistical significance was determined with a Monte Carlo simulation. The accuracy of synergism between Pfcrt and Pfmdr1 mutant genes was also tested using the Receiver Operating Characteristic (ROC) curve.

Results

Prevalence of point mutations in Pfmdr1 and Pfcrt gene of the *P. falciparum* isolates

Two hundred and fourteen *P. falciparum* isolates were analyzed for detection of mutations at N86Y, Y184F, S1034C, D1042N, and D1246Y of the Pfmdr1 gene and at codon 76 (K76T) of the Pfcrt gene. The prevalence of Y86, F184, C1034, Y1246 mutations of the Pfmdr1 gene determined were 36.0%, 87.9%, 71.0%, 91.6% and 8.4%, respectively (Table 1). Around half (53.7%) of the examined isolates had the T76 mutation of the Pfcrt gene (Table 1). The prevalence of Pfmdr1 haplotypes in the *P. falciparum* isolates were mainly NFCDD ($n = 93$, 43.46%), YFCDD ($n = 59$, 27.57%), NFSDD ($n = 16$, 7.48%), NYSNY ($n = 11$, 5.14%) and YFSDD ($n = 10$, 4.67%; Tables S1 and S2).

Co-existence of Pfcrt and Pfmdr1 genes

Table 2 shows the association of Pfcrt alleles at codon 76 with alleles of Pfmdr1 at codon 86, 184, 1034, 1042 and 1246. One hundred thirty-seven of the total number of isolates examined had the wild-type allele at codon 86 of the Pfmdr1 gene together with the Pfcrt gene, while 29.2% had K76, 55.5% had T76 and 15.3% had both the K76 and T76 alleles. There was no significant difference between the codon 76 polymorphisms of the Pfcrt gene and the wild-type allele at codon 86 of the

Table 2 Association of Pfcrt alleles at codon 76 with alleles of Pfmdr1 at codon 86, 184, 1034, 1042 and 1246.

	Pfcrt	Pfmdr1											
Codon	76	86		184		1034		1042		1246			
Single nucleotide polymorphisms *N* (%)		N	Y	Y	F	S	C	N	D	D	Y	D/Y	
	K	40 (29.2)	20 (26.0)	7 (26.9)	53 (28.2)	16 (25.8)	44 (28.9)	5 (27.8)	55 (28.1)	49 (27.4)	7 (38.9)	4 (23.5)	
	T	76 (55.5)	39 (56.6)	11 (42.3)	104 (55.3)	27 (43.5)	88 (57.9)	6 (33.3)	109 (55.6)	102 (57.0)	6 (33.3)	7 (41.2)	
	K/T	21 (15.3)	18 (23.4)	8 (30.8)	31 (16.5)	19 (30.6)	20 (13.2)	7 (38.9)	32 (16.3)	28 (15.6)	5 (27.8)	6 (35.3)	
Total		137	77	26	188	62	152	18	196	179	18	17	
Pearson χ^2		10.961	19.506	6.186	22.747	10.880	23.815	11.892	20.494	22.792	5.817	7.083	
p-Value		0.090	**0.003****	0.403	**0.001****	0.092	**0.001****	0.064	**0.002****	**0.001****	0.444	0.313	

* significant ($p < 0.05$); ** significant ($p < 0.01$); *** highly significant ($p < 0.001$).

Table 3 Association of Pfcrt 76T mutation with the double mutation polymorphisms of the Pfmdr1 gene.

Codon polymorphisms		Pearson χ^2	*p*-Value
Pfmdr1 86 and 184	Pfcrt 76		
NY	**T**		
NY	K	4.083	0.665
YY	**T**		
YY	K	6.667	0.155
NF	**T**		
NF	K	8.130	0.229
YF	**T**		
YF	K	18.045	**0.006*****

NB: The bold and underlined amino acids are mutant alleles.
* significant ($p < 0.05$); ** significant ($p < 0.01$); *** highly significant ($p < 0.001$).

Pfmdr1 gene ($\chi^2 = 10.961$, $p = 0.090$). Seventy-seven (77/214) of the isolates had the mutant allele at codon 86 of Pfmdr1. A statistically significant difference was observed between the isolates with a mutation at codon 86 of Pfmdr1 co-existing with that at codon 76 of Pfcrt (mutant allele) compared to isolates with a mutant allele at codon 86 of Pfmdr1 co-existing with wild-type allele at codon 76 of the Pfcrt gene ($\chi^2 = 19.506$, $p = 0.003$). Similar trends were observed between the mutations at codon 184, 1034 and 1042 of the Pfmdr1 and the mutant Pfcrt gene. Observations made in this study indicated an association between a Pfcrt 76T mutation with multiple mutations at 86, 184, 86, 184 and 1034 codons of the Pfmdr1 gene. The association between Pfcrt T76 and double and triple mutations at 86, 184 and 1034 codon positions of the Pfmdr1 gene was also discovered. The results shown in Table 3 indicate a significant association between Pfcrt T76 and Y86-F184 haplotype of Pfmdr1 ($\chi^2 = 18.045$, $p = 0.006$) but not the NY, YY or NF haplotypes of the Pfmdr1 isolates when only polymorphisms at codon 86 and 184 of Pfmdr1 were considered. The test of association between Pfcrt T76 and the triple codon haplotype mutations of Pfmdr1 showed a similar trend to that observed for the double codon polymorphisms. Two levels of association were observed for the triple haplotype at 86, 184 and 1034 of Pfmdr1 and Pfcrt T76, NFC ($\chi^2 = 13.770$, $p = 0.032$) and YFC ($\chi^2 = 16.489$, $p = 0.011$), but not with YFS ($\chi^2 = 5.511$, $p = 0.480$; Table 4). The pattern of association did not change when haplotypes for 86, 184, 1034 and 1042 of Pfmdr1 were determined. Similarly, haplotypes for 86, 184, 1034, 1042 and 1246 of Pfmdr1 showed an association with Pfcrt T76 as did the triple haplotypes of Pfmdr1: NFCDD ($\chi^2 = 13.770$, $p = 0.032$) and YFCDD ($\chi^2 = 16.487$,

Table 4 Association of Pfcrt 76T mutation with the triple mutations polymorphism of the Pfmdr1 gene.

Codon polymorphisms		Pearson χ^2	*p*-Value
Pfmdr1 86, 184 and 1034	Pfcrt 76		
NYS	**T**		
NYS	K	4.083	0.665
YYS	**T**		
YYS	K	6.667	0.155
N**F**S	**T**		
N**F**S	K	7.157	0.307
YFS	**T**		
YFS	K	5.511	0.480
N**FC**	**T**		
N**FC**	K	13.770	**0.032***
YFC	**T**		
YFC	K	16.489	**0.011****

NB: The bold and underlined amino acids are mutant alleles. * significant ($p < 0.05$); ** significant ($p < 0.01$); *** highly significant ($p < 0.001$).

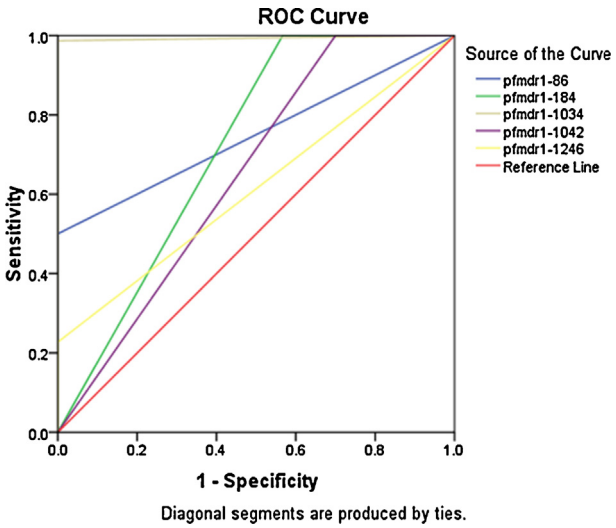

Figure 2 ROC curve for true positive synergism rate against false positive synergism between Pfcrt and Pfmdr1 genes. The diagonal segment is ties produced by Pfcrt 76 polymorphism.

$p = 0.011$). No association was observed for YYSNY ($\chi^2 = 5.000$, $p = 0.287$; Tables S1 and S2).

Synergism between Pfcrt and Pfmdr1 gene

The co-existence between the T76 mutation of Pfcrt gene and mutations at codon positions 86, 184, and 1034 of Pfmdr1 were further tested for stochastic prevalence using the Jonckheere—Terpstra test (J—T). The standard J—T statistic between the Pfcrt at codon 76 polymorphism and Pfmdr1 at codon positions 86, 184, 1034, 1042 and 1246 were 6.830, 8.695, 14.264, 7.086 and 4.028, respectively, with $p < 0.0001$. A significant synergism ($p < 0.0001$) between Pfcrt 76 mutation and the five point mutations of Pfmdr1 were also observed using Monte Carlo simulation at a 99% confidence interval using the Pfcrt 76 polymorphism as a group variable (Table 5). Accuracy of the synergism between the Pfcrt and Pfmdr1 genes was tested again using the Receiver Operating characteristic (ROC) curve. The analysis indicated synergism between the Pfcrt 76 mutation and Pfmdr1-1034 mutation perfect (area under ROC curve = 0.994 ± 0.005, $p < 0.0001$) while that between Pfcrt 76 and Pfmdr1 at codon positions 86, 184, 1042 and 1246 were also significant, with a ROC area of 0.750 ± 0.032, 0.717 ± 0.045, 0.650 ± 0.046 and 0.614 ± 0.039, respectively. However, synergism between Pfcrt 76 and Pfmdr1-1042 ($p = 0.001$) and Pfmdr1-1246 ($p = 0.01$) were observed to be comparatively weaker (Fig. 2 and Table 6).

Discussion

The association between chloroquine treatment failure and K76T mutation in Pfcrt is well-known [25,26]. The contribution of N86Y and D1246Y mutations of Pfmdr1 to the fitness of CQ resistance *P. falciparum* strains has also been documented [27,28]. The withdrawal of CQ from Malawi, Mozambique and Kenya led to a rapid recovery of CQ sensitive *P. falciparum*, which increased the limited fitness costs that accompany CQ resistance [29,30]. However, a rapid recovery of CQ sensitive strains was not observed within the same time frame in other disease endemic countries, such as Ghana and Uganda after CQ withdrawal [10—12,14,31]. Thus, the principle objective for this study became ascertaining reasons for the slow recovery of CQ sensitive *P. falciparum* with an increasing presence of amodiaquine and quinine resistance in Ghana. We have previously shown that the CQ resistance marker Pfcrt T76 remains very high in Ghana even after several years of CQ withdrawal [10,11]. In the current study we demonstrated an association of the chloroquine resistance marker with all the point mutations in Pfmdr1; at codon 86, 184, 1034 and 1042, except at codon 1246. This finding may explain the continuous presence of significant levels of CQ resistant parasites in Ghana.

The mutations of Pfmdr1 gene are complex and the mechanism for modulating *P. falciparum* multiple antimalarial drug resistance is not well-understood [32,33]. The effect of this mutation changes unpredictably, as different mutations

Table 5 Synergism between the Pfcrt and Pfmdr1 gene polymorphism.

Jonckheere—Terpstra test						
			Pfmdr1-86	Pfmdr1-184	Pfmdr1-1034	Pfmdr1-1042	Pfmdr1-1246
Number of levels in Pfcrt-76			2	2	2	2	2
N			214	214	214	214	214
Observed J—T statistic			6930.00	6622.00	9180.00	6006.00	5670.00
Mean J—T statistic			4620.00	4620.00	4620.00	4620.00	4620.00
Std. deviation of J—T statistic			338.238	230.241	319.694	195.605	260.661
Std. J—T statistic			6.830	8.695	14.264	7.086	4.028
Asymp. sig. (2-tailed)			0.0001	0.0001	0.0001	0.0001	0.0001
Monte Carlo sig.	Sig.		0.0001	0.0001	0.0001	0.0001	0.0001
(2-tailed)	99% CI	Lower bound	0.0	0.0	0.0	0.0	0.0
		Upper bound	0.0	0.0	0.0	0.0	0.001
Monte Carlo sig.	99% CI	Lower bound	0.0	0.0	0.0	0.0	0.0
(1-tailed)		Upper bound	0.0	0.0	0.0	0.0	0.0
	Sig.		0.0001	0.0001	0.0001	0.0001	0.0001

Table 6 Area under the synergism ROC curve.

Variable(s)	Area ± SE[a]	Asymptotic 95% CI	Asymptotic *p*-Value[b]
Pfmdr1-86	0.750 ± 0.032	0.687—0.813	0.0001
Pfmdr1-184	0.717 ± 0.045	0.629—0.804	0.0001
Pfmdr1-1034	0.994 ± 0.005	0.983—1.004	0.0001
Pfmdr1-1042	0.650 ± 0.046	0.560—0.740	0.001
Pfmdr1-1246	0.614 ± 0.039	0.537—0.691	0.010

The test result variable(s): Pfmdr1-86, Pfmdr1-184, Pfmdr1-1034, Pfmdr1-1042, and Pfmdr1-1246 have at least one tie between the positive actual state group and the negative actual state group.

[a] Under the nonparametric assumption.

[b] Null hypothesis: true area = 0.5.

can increase the parasite's susceptibility toward one antimalarial drug or simultaneously confer resistance to another [34,35]. For instance, the N86Y-Y184F-D1246Y mutation of Pfmdr1 polymorphism causes *P. falciparum* to be susceptible to artemisinin-based combination therapy, whereas NFCDY or NFSDD haplotypes at codon 86, 184, 1034, 1042, and 1246 are associated with AQ failure in South America [36—39].

To understand the complexities of mutant Pfmdr1 genes and their relationship with the mutant Pfcrt gene at codon 76, we tested for synergism between them using statistical methods. The results indicated a strong synergic interaction between Pfcrt 76 polymorphism and that of Pfmdr1 at 86, 184, 1034, 1042 and 1246. When the accuracy of the synergy was verified using ROC, the outcome showed a stronger synergy between Pfcrt 76 polymorphism and that of Pfmdr1 at codon 1034, 86, and 184 but was comparatively weaker with that at codon 1042 and 1246.

Amodiaquine and quinine are important antimalarial drugs in Ghana. The former is a co-partner in the first line of treatment of uncomplicated malaria using ACT, and the latter is the drug of choice for the management of complicated malaria. However, recent reports indicate some degree of parasite resistance to these drugs. In an attempt to understand the mechanism of resistance to these drugs in Ghana, we examined the association between Pfcrt K76T and double mutational haplotypes at 86 and 184 of the Pfmdr1 gene. The results showed that Pfcrt T76 was strongly associated with the double mutation YF haplotype but not in NY, YY or NF. This unique association has not been previously reported, although individually the 86Y of Pfmdr1 has been reported to be associated with CQ or amodiaquine resistance, whereas 184F and 1042D have been shown to have less sensitivity to QN [40,41]. Double mutation at 184 and 1042 of Pfmdr1 gene has been reported to reduce *P. falciparum* sensitivity to quinine whereas mutation at 86 and 1042 increases the parasite's susceptibility to quinine treatment [42,43]. This means that modulation of quinine resistance could depend on specific combinations of mutations of Pfmdr1. For instance, a single mutation at codon 86 has been shown to increase *P. falciparum* susceptibility to quinine

[43]. However, a N1042D mutation of Pfmdr1 can either increase or reduce the parasite's susceptibility to quinine [42]. Furthermore, the insertion or deletion of haplotype mutations 1034C, 1042D and 1246Y of Pfmdr1 resulted in either quinine-resistance or quinine sensitive *P. falciparum* strains [43]. This tends to suggest that the Pfcrt T76 & YFC haplotype of Pfmdr1 could contribute to the emergence of quinine resistance in Ghana. Although quinine remains the antimalarial drug of choice for the management of complicated malaria in Ghana, the recent report of an elevated IC_{50} value [13,14] for the drug, in addition to the observations made in this study, should be a matter of concern to stakeholders.

From the data gathered in this study, it is reasonable to assert the association of Pfcrt-T76 with a N86-F184-C1034 mutation of Pfmdr1 may be responsible for the presence of amodiaquine resistance in Ghana. This assertion is justified by field-base report of strains of *P. falciparum* carrying either CVIET or SVMNT at codon 72—76 of the Pfcrt gene and NFCDY or NFSDD at codon 86, 184, 1034, 1042, 1246 of the Pfmdr1 gene [42], as well as an association with high in vitro IC_{50} values for monodesethylamodiaquine.

P. falciparum strains with the N86, F184 and D1246 (NFD) haplotype have been reported to have a fitness advantage under artemether-lumefantrine (AL) pressure [39]. Thus, the observed increase in prevalence of NFD in Tanzania and Mozambique after the introduction of AL could involve NFD under AL pressure. Based on observations made in this study, is possible artemether-lumefantrine resistant *P. falciparum* may emerge in Ghana in the near future. The reported high variation in patient response to treatment with artemether-lumefantrine in different ecological zones [41] could signify an onset of resistance to the combination antimalarial drug.

Several reports have shown that integrated vector control strategies, such as the use of long lasting insecticide-treated nets (LLIN) and indoor residual spraying (IRS), have resulted in reduced malaria morbidity and mortality especially among children [44—46]. In Ghana, vector control programs have been in place since 2005 but have not had any significant impact on disease prevalence until a recent refocus of efforts. New efforts involve not only free distribution of LLIN, but a door-to-door campaign to hang these nets by community-based volunteers to enhance usage. Additionally, indoor residual spraying in communities, including the study areas, has been ongoing. These interventional measures appear to have positive impact, as latest reports from the NMCP indicate the malaria prevalence rate is declining in Ghana [47]. Despite the clear public health benefit of the vector control measures, the impact of these measures on the spread of drug resistance *P. falcipaum* is not well understood. In 2011, Shi et al. reported there is no evidence to suggest sustained transmission reduction by LLIN usage reduces the prevalence of the genes associated with malaria drug resistance [48]. The influence of these vector control measures on the study findings could not be immediately determined.

Observations made in this study strongly suggest the slow recovery of chloroquine sensitive *P. falciparum* strains and the presence of amodiaquine and quinine resistance in Ghana could be due to synergism between Pfcrt and Pfmdr1 mutant genes. It must be emphasized that this could be caused by indiscriminate use of CQ with the resultant selection for resistant strains of the parasite. Chloroquine is a safe and affordable drug, and with gains in efficacy, could be adapted for use as one of the partners in a drug combination therapy. It is therefore imperative to put measures in place to ensure the fast restoration of chloroquine use in Ghana.

Conclusions

Findings from this study suggest synergism between mutant Pfcrt and Pfmdr1 genes in *P. falciparum* could account for the slow recovery of chloroquine sensitive parasites long after the disuse of chloroquine in Ghana. This phenomenon could also be responsible for the presence of quinine and amodiaquine resistance in the country. From the gathered data, if the right preventive measures are not put in place, artemether-lumefantrine resistance could emerge in Ghana sooner rather than later.

Funding

No funding sources.

Competing interests

None declared.

Ethical approval

The study proposal and protocol was approved by the Ghana Health Service Ethics Committee

(GHS-ERC-17/01/12) and University of Cape Coast Institutional Review Board (UCCIRB/28/09/3.1.1).

Acknowledgments

This work was supported with funds from the Department of Biomedical and Forensic Sciences, University of Cape Coast, Cape Coast. We are grateful to the laboratory and the medical staff of St. Francis Xavier hospital, Cape Coast Metropolitan hospital, the Twifo Praso district hospital and the Elmina Urban Health Center for technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jiph.2016.02.004.

References

[1] Mekonnen SK, Aseffa A, Berhe N, Teklehaymanot T, Clouse RM, Gebru T, et al. Return of chloroquine-sensitive *Plasmodium falciparum* parasites and emergence of chloroquine-resistant *Plasmodium vivax* in Ethiopia. Malar J 2014;13:244.

[2] Gharbi M, Flegg JA, Hubert V, Kendjo E, Metcalf JE, Bertaux L, et al. Longitudinal study assessing the return of chloroquine susceptibility of *Plasmodium falciparum* in isolates from travellers returning from West and Central Africa, 2000—2011. Malar J 2013;12:1.

[3] Mohammed A, Ndaro A, Kalinga A, Manjurano A, Mosha JF, Mosha DF, et al. Trends in chloroquine resistance marker, Pfcrt-K76T mutation ten years after chloroquine withdrawal in Tanzania. Malar J 2013;12:415.

[4] Laufer MK, Takala-Harrison S, Dzinjalamala FK, Stine OC, Taylor TE, Plowe CV. Return of chloroquine-susceptible falciparum malaria in Malawi was a reexpansion of diverse susceptible parasites. J Infect Dis 2010;202:801—8.

[5] Gresty KJ, Gray KA, Bobogare A, Taleo G, Hii J, Wini L, et al. Genetic mutations in pfcrt and pfmdr1 at the time of artemisinin combination therapy introduction in South Pacific islands of Vanuatu and Solomon Islands. Malar J 2014;13:406.

[6] Ibraheem ZO, Abd Majid R, Noor SM, Sedik HM, Basir R. Role of different pfcrt and pfmdr-1 mutations in conferring resistance to antimalaria drugs in *Plasmodium falciparum*. Malar Res Treat 2014;2014:950424.

[7] Shrivastava SK, Gupta RK, Mahanta J, Dubey ML. Correlation of molecular markers, Pfmdr1-N86Y and Pfcrt-K76T, with in vitro chloroquine resistant *Plasmodium falciparum*, isolated in the malaria endemic states of Assam and Arunachal Pradesh, Northeast India. PLOS ONE 2014;9:e103848.

[8] Folarin OA, Gbotosho GO, Sowunmi A, Olorunsogo OO, Oduola AMJ, Happi TC. Chloroquine resistant *Plasmodium falciparum* in Nigeria: relationship between pfcrt and pfmdr1 polymorphisms, in-vitro resistance and treatment outcome. Open Trop Med J 2008;1:74—82.

[9] Kebede S, Medhin G, Berhe N, Teklehaymanot T, Gebru T, Clause R, et al. Return of chloroquine-sensitive *Plasmodium falciparum* parasites and emergence of chloroquine-resistant *Plasmodium vivax* in Ethiopia. Malar J 2014;13:1.

[10] Asare KK, Boampong JN, Afoakwah R, Ameyaw EO, Sehgal R, Quashie NB. Use of proscribed chloroquine is associated with an increased risk of pfcrt T76 mutation in some parts of Ghana. Malar J 2014;13:246.

[11] Afoakwah R, Boampong JN, Egyir-Yawson A, Nwaefunab EK, Verner ON, Asare KK. High prevalence of PfCRT K76T mutation in *Plasmodium falciparum* isolates in Ghana. Acta Trop 2014;136:32—6.

[12] Duah NO, Matrevi SA, de Souza DK, Binnah DD, Tamakloe MM, Opoku VS, et al. Increased pfmdr1 gene copy number and the decline in pfcrt and pfmdr1 resistance alleles in Ghanaian *Plasmodium falciparum* isolates after the change of anti-malarial drug treatment policy. Malar J 2013;12:377.

[13] Quashie NB, Duah NO, Abuaku B, Quaye L, Ayanful-Torgby R, Akwoviah GA, et al. A SYBR Green 1-based in vitro test of susceptibility of Ghanaian *Plasmodium falciparum* clinical isolates to a panel of anti-malarial drugs. Malar J 2013;12:450.

[14] Kwansa-Bentum B, Ayi I, Suzuki T, Otchere J, Kumagai T, Anyan WK, et al. *Plasmodium falciparum* isolates from southern Ghana exhibit polymorphisms in the SERCA-type PfATPase6 though sensitive to artesunate in vitro. Malar J 2011;10:187.

[15] Das S, Mahapatra SK, Tripathy S, Chattopadhyay S, Dash SK, Mandal D, et al. Double mutation in the pfmdr1 gene is associated with emergence of chloroquine-resistant *Plasmodium falciparum* malaria in Eastern India. Antimicrob Agents Chemother 2014;58:5909—15.

[16] Sidhu ABS, Valderramos SG, Fidock DA. pfmdr1 mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. Mol microbial 2005;57:913—26.

[17] Griffing S, Syphard L, Sridaran S, McCollum AM, Mixson-Hayden T, Vinayak S, et al. pfmdr1 amplification and fixation of pfcrt chloroquine resistance alleles in *Plasmodium falciparum* in Venezuela. Antimicrob Agents Chemother 2010;54:1572—9.

[18] Zakeri S, Afsharpad M, Kazemzadeh T, Mehdizadeh K, Shabani A, Djadid ND. Association of pfcrt but not pfmdr1 alleles with chloroquine resistance in Iranian isolates of *Plasmodium falciparum*. Am J Trop Med Hyg 2008;78:633—40.

[19] Plummer WB, Pereira LMP, Carrington CV. Pfcrt and pfmdr1 alleles associated with chloroquine resistance in *Plasmodium falciparum* from Guyana, South America. Mem Inst Oswaldo Cruz 2004;99:389—92.

[20] Sidhu ABS, Uhlemann AC, Valderramos SG, Valderramos JC, Krishna S, Fidock DA. Decreasing pfmdr1 copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. J Infect Dis 2006;194:528—35.

[21] Humphreys GS, Merinopoulos I, Ahmed J, Whitty CJM, Mutabingwa TK, Sutherland CJ, et al. Amodiaquine and artemether-lumefantrine select distinct alleles of the *Plasmodium falciparum* mdr1 gene in Tanzanian children treated for uncomplicated malaria. Antimicrob Agents Chemother 2007;51:991—7.

[22] Sisowath C, Ferreira PE, Bustamante LY, Dahlström S, Mårtensson A, Björkman A, et al. The role of pfmdr1 in *Plasmodium falciparum* tolerance to artemether-lumefantrine in Africa. Trop Med Int Health 2007;12:736—42.

[23] Chen N, Russell B, Fowler E, Peters J, Cheng Q. Levels of chloroquine resistance in *Plasmodium falciparum* are

determined by loci other than pfcrt and pfmdr1. J Infect Dis 2002;185:405—7.
[24] Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, et al. A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med 2001;344:257—63.
[25] Baro NK, Callaghan PS, Roepe PD. Function of resistance conferring *Plasmodium falciparum* chloroquine resistance transporter isoforms. Biochemistry 2013;52:4242—9.
[26] Valderramos SG, Valderramos JC, Musset L, Purcell LA, Mercereau-Puijalon O, Legrand E, et al. Identification of a mutant PfCRT-mediated chloroquine tolerance phenotype in *Plasmodium falciparum*. PLoS Pathog 2010;6:e1000887.
[27] Ochong E, Tumwebaze PK, Byaruhanga O, Greenhouse B, Rosenthal PJ. Fitness consequences of *Plasmodium falciparum* pfmdr1 polymorphisms inferred from ex vivo culture of Ugandan parasites. Antimicrob Agents Chemother 2013;57:4245—51.
[28] Yang Z, Zhang Z, Sun X, Wan W, Cu L, Zhang X, et al. Molecular analysis of chloroquine resistance in *Plasmodium falciparum* in Yunnan Province, China. Trop Med Int Health 2007;12:1051—60.
[29] Petersen I, Gabryszewski SJ, Johnston GL, Dhingra SK, Ecker A, Lewis RE, et al. Balancing drug resistance and growth rates via compensatory mutations in the *Plasmodium falciparum* chloroquine resistance transporter. Mol Microbiol 2015.
[30] Jiang H, Patel JJ, Yi M, Mu J, Ding J, Stephens R, et al. Genome-wide compensatory changes accompany drug-selected mutations in the *Plasmodium falciparum* crt gene. PLoS ONE 2008;3:e2484.
[31] Kamugisha E, Bujila I, Lahdo M, Pello-Esso S, Minde M, Kongola G, et al. Large differences in prevalence of Pfcrt and Pfmdr1 mutations between Mwanza, Tanzania and Iganga, Uganda — a reflection of differences in policies regarding withdrawal of chloroquine. Acta Trop 2012;12:148—51.
[32] Pelleau S, Bertaux L, Briolant S, Ferdig MT, Sinou V, Pradines B, et al. Differential association of *Plasmodium falciparum* Na+/H+ exchanger polymorphism and quinine responses in field- and culture-adapted isolates of *Plasmodium falciparum*. Antimicrob Agents Chemother 2011;55:5834—41.
[33] Roepe PD. The molecular physiologic basis of quinoline drug resistance in *P. falciparum* malaria. Future Microbiol 2009;4:441—55.
[34] Mbaisi A, Liyala P, Eyase F, Achilla R, Akala H, Wangui J, et al. Drug susceptibility and genetic evaluation of *Plasmodium falciparum* isolates obtained in four distinct geographical regions of Kenya. Antimicrob Agents Chemother 2004;48:3598—601.
[35] Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, et al. Mefloquine resistance in *Plasmodium falciparum* and increased pfmdr1 gene copy number. Lancet 2004;364:438—47.
[36] Lobo E, de Sousa B, Rosa S, Figueiredo P, Lobo L, Pateira S, et al. Prevalence of pfmdr1 alleles associated with artemether-lumefantrine tolerance/resistance in Maputo before and after the implementation of artemisinin-based combination therapy. Malar J 2014;13:300.
[37] Baliraine FN, Rosenthal PJ. Prolonged selection of pfmdr1 polymorphisms after treatment of falciparum malaria with artemether-lumefantrine in Uganda. J Infect Dis 2011;204:1120—4.
[38] Thomsen TT, Ishengoma DS, Mmbando BP, Lusingu JP, Vestergaard LS, Theander TG, et al. Prevalence of single nucleotide polymorphisms in the *Plasmodium falciparum* multidrug resistance gene (Pfmdr-1) in Korogwe District in Tanzania before and after introduction of artemisinin-based combination therapy. Am J Trop Med Hyg 2011;85:979—83.
[39] Wong RP, Karunajeewa H, Mueller I, Siba P, Zimmerman PA, Davis TM. Molecular assessment of *Plasmodium falciparum* resistance to antimalarial drugs in Papua New Guinea using an extended ligase detection reaction fluorescent microsphere assay. Antimicrob Agents Chemother 2011;55:798—805.
[40] Eyase FL, Akala HM, Ingasia L, Cheruiyot A, Omondi A, Okudo C, et al. The role of Pfmdr1 and Pfcrt in changing chloroquine, amodiaquine, mefloquine and lumefantrine susceptibility in Western-Kenya *P. falciparum* samples during 2008—2011. PLoS ONE 2013;8:e64299.
[41] Abuaku B, Duah N, Quaye L, Quashie N, Koram K. Therapeutic efficacy of artemether-lumefantrine combination in the treatment of uncomplicated malaria among children under five years of age in three ecological zones in Ghana. Malar J 2012;11:388.
[42] Gadalla NB, Tavera G, Mu J, Kabyemela ER, Fried M, Duffy PE, et al. Prevalence of *Plasmodium falciparum* anti-malarial resistance-associated polymorphisms in pfcrt, pfmdr1 and pfnhe1 in Muheza, Tanzania, prior to introduction of artemisinin combination therapy. Malar J 2015;14:129.
[43] Wurtz N, Fall B, Pascual A, Fall M, Baret E, Camara C, et al. Role of Pfmdr1 in in vitro *Plasmodium falciparum* susceptibility to chloroquine, quinine, monodesethylamodiaquine, mefloquine, lumefantrine, and dihydroartemisinin. Antimicrob Agents Chemother 2014;58:7032—40.
[44] Fullman N, Burstein R, Lim SS, Medlin C, Gakidou E. Nets, spray or both? The effectiveness of insecticide-treated nets and indoor residual spraying in reducing malaria morbidity and child mortality in sub-Saharan Africa. Malar J 2013;12(62):10—86.
[45] Hamel MJ, Otieno P, Bayoh N, Kariuki S, Were V, Marwanga D, et al. The combination of indoor residual spraying and insecticide-treated nets provides added protection against malaria compared with insecticide-treated nets alone. Am J Trop Med Hyg 2011;85(6):1080—6.
[46] West PA, Protopopoff N, Wright A, Kivaju Z, Tigererwa R, Mosha F, et al. Indoor residual spraying in combination with insecticide-treated nets compared to insecticide-treated nets alone for protection against malaria: a cluster randomised trial in Tanzania. PLoS Med 2014;11(4):e1001630.
[47] http://www.newsghana.com.gh/ghana-makes-positive-progress-in-fighting-malaria-as-prevalence-rate-declines/.
[48] Shah M, Kariuki S, Eng JV, Blackstock AJ, Garner K, Gatei W, et al. Effect of transmission reduction by insecticide-treated bednets (ITNs) on antimalarial drug resistance in Western Kenya. PLoS ONE 2011;6(11):e26746.