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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

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Abstract

Background: With the introduction of artemisinin-based combination therapy (ACT) in 2005, monitoring of anti-malarial drug efficacy, which includes the use of molecular tools to detect known genetic markers of parasite resistance, is important for first-hand information on the changes in parasite susceptibility to drugs in Ghana. This study investigated the *Plasmodium falciparum* multidrug resistance gene (*pfmdr1*) copy number, mutations and the chloroquine resistance transporter gene (*pfcrt*) mutations in Ghanaian isolates collected in seven years to detect the trends in prevalence of mutations.

Methods: Archived filter paper blood blots collected from children aged below five years with uncomplicated malaria in 2003–2010 at sentinel sites were used. Using quantitative real-time polymerase chain reaction (qRT-PCR), 756 samples were assessed for *pfmdr1* gene copy number. PCR and restriction fragment length polymorphism (RFLP) were used to detect alleles of *pfmdr1* 86 in 1,102 samples, *pfmdr1* 184, 1034, 1042 and 1246 in 832 samples and *pfcrt* 76 in 1,063 samples. Merozoite surface protein 2 (*msp2*) genotyping was done to select monoclonal infections for copy number analysis.

Results: The percentage of isolates with increased *pfmdr1* copy number were 4, 27, 9, and 18% for 2003–04, 2005–06, 2007–08 and 2010, respectively. Significant increasing trends for prevalence of *pfmdr1* N86 ($x^2 = 96.31$, p <0.001) and *pfcrt* K76 ($x^2 = 64.50$, p <0.001) and decreasing trends in *pfmdr1* Y86 ($x^2 = 38.52$, p <0.001) and *pfcrt* T76 ($x^2 = 43.49$, p <0.001) were observed from 2003–2010. The *pfmdr1* F184 and Y184 prevalence showed an increasing and decreasing trends respectively but were not significant ($x^2 = 7.39$, p=0.060; $x^2 = 7.49$, p = 0.057 respectively). The *pfmdr1* N86-F184-D1246 haplotype, which is alleged to be selected by artemether-lumefantrine showed a significant increasing trend ($x^2 = 20.75$, p < 0.001).

Conclusion: Increased *pfmdr1* gene copy number was observed in the isolates analysed and this finding has implications for the use of ACT in the country although no resistance has been reported. The decreasing trend in the prevalence of chloroquine resistance markers after change of treatment policy presents the possibility for future introduction of chloroquine as prophylaxis for malaria risk groups such as children and pregnant women in Ghana.

Keywords: Anti-malarial drug resistance, *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt*), *Plasmodium falciparum* multidrug resistance gene (*pfmdr*1), Molecular markers, Ghana

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Background

Malaria is a childhood killer disease in sub-Saharan Africa. Consequently, the absence of an effective malaria vaccine and low uptake of currently available preventive tools makes chemotherapy a strong pillar of the strategies to control malaria in disease-endemic areas of the world. However, the emergence and spread of clones of *Plasmodium falciparum* which are resistant to most available anti-malarial drugs makes the control of the disease difficult to achieve. Since the first report of chloroquine-resistant *P. falciparum* in Thailand [1] and the rapid spread of resistance worldwide, followed by the recommendation by WHO to use artemisinin-based combination therapy (ACT), many countries in malaria-endemic areas have been monitoring anti-malarial drug resistance. Active implementation of malaria surveillance and the exploitation of molecular as well as phenotypic methods to characterize parasite drug susceptibility profiles will expedite the clarification of spatio-temporal drug sensitivity. This will enable early detection of resistance to the artemisinin derivatives and more efficient use of other available anti-malarial drugs.

Chloroquine resistance has been linked to single nucleotide polymorphisms (SNPs) in the *P. falciparum* transporter gene (*pfcrt*) on chromosome 7 [2]. The level of parasite resistance to this drug is under multilocus/multigenic control [3]. Sets of SNPs in *pfcrt* codons 72, 74, 75, 76, 97, 152, 163, 220, 271, 326, 356 and 371 were associated with chloroquine resistance in *P. falciparum* from Southeast Asia, Africa and South America [4-6]. The mutation on codon 76, that results from the substitution of threonine for lysine in the gene sequence is the seminal SNP for producing the resistance phenotype and the most reliable molecular marker of chloroquine resistance among the various mutations identified [5,6]. In addition there is reported link between T76 and AQ resistance, however the association is comparatively weaker to that of the other aminoquinoline chloroquine [7-11]. Another genetic mechanism of chloroquine resistance is the SNPs in the *P. falciparum* multidrug-resistance gene (*pfmdr*1) on chromosome 5 which encodes a P-glycoprotein homologue-1 multi-drug resistant transporter located in the parasite food vacuole, and is associated with enhanced efflux of the drug from resistant parasites [12]. The *pfmdr*1 mutations linked to anti-malarial drug resistance occur at codons 86, 184, 1034, 1042 and 1246 [12-15]. However, the mutation that occurs as a result of the substitution of asparagine for tyrosine at position 86 is linked with chloroquine resistance [12,14,16-19]. Additionally, multiple *pfmdr*1 SNPs have been associated with susceptibility profiles of many anti-malarial drugs. These SNPs, as well as the gene amplification, may alter substrate specificity for aminoquinolines and arylaminoalcohols [20] promoting *P. falciparum*

resistance to diverse anti-malarials, including chloroquine, mefloquine, quinine and artemisinin derivatives. The Y184F, N1042D and D1246Y mutations are associated with the chloroquine resistance phenotype from samples in Africa, Asia and South America [3,21,22]. The *pfmdr*1 haplotype of N86, F184 and D1246 (NFD) were selected in recrudescence samples after artemether-lumefantrine (AL) treatment suggesting that this haplotype conferred a fitness advantage upon AL pressure [23]. With the introduction of AL use in Tanzania and Mozambique, the trends in NFD prevalence have been shown to be increasing [24-26] which is supports the fact of AL pressure. In addition, the *pfmdr*1 N86 and *pfcrt* K76 wild-type alleles are also selected by AL treatment but this observation was not made in the use of artesunate-amodiaquine (AS-AQ) and amodiaquine-sulphadoxine-pyrimethamine (AQ-SP) [27]. The *pfmdr*1 D1246Y and N86Y mutations predict resistance and recrudescence to AQ and quinine in Uganda [28]. The N86Y mutation increases sensitivity to artemisinin and dihydroartemisinin-piperaquine (DHAP) [20]. The N86Y, Y184F and N1042D mutations increased susceptibility to aryl-amino-alcohol drugs, including mefloquine, halofantrine, lumefantrine, and artemisinin derivatives [28-32]. In South America, the S1034C, N1042D and D1246Y mutations are associated with quinine resistance [33] and are also implicated in increased sensitivity to artemisinin [34,35]. The S1034C and N1042D mutations were reported to reduce or abolish resistance to mefloquine [30].

The *pfmdr*1 gene expression levels have been considered in the etiology of the parasite resistance to some anti-malarial drugs and it is being explored in epidemiological studies. Increase in *pfmdr*1 gene copy number has been linked to *P. falciparum* diminished susceptibility to anti-malarial drugs, such as mefloquine, AS-MQ and AL combinations [36-38]. Although there is no reported correlation of *pfmdr*1 gene copy number and treatment failure, this marker is important for the prediction of recrudescence with the use of the anti-malarials mentioned above [39]. There is an assertion that *pfmdr*1 gene copy number rather than the SNPs exercises greater influence in mediating anti-malarial drug resistance to some compounds [40]. This was reported to be due to the fact that many transporter proteins mandate concerted complementary attention to copy number variations (CNV) in mediating anti-malarial activity [40].

Ghana is a malaria-endemic country using ACT (AS-AQ, AL and DHAP) for the treatment of uncomplicated malaria since 2005. Information from molecular investigations, in addition to *in vivo* and *in vitro* analysis, is crucial for early detection and prediction of resistance to the ACT. Before the change of the drug policy in Ghana, the prevalence of the *pfcrt* T76 mutation and the *pfmdr*1 Y86 mutation associated with chloroquine resistance ranged

from 46 to 98% and 42 to 95%, respectively from five sentinel sites [16]. Due to the Malawian and Kenyan experiences where there was significant decrease in the resistant parasite populations when the drug was withdrawn from use [41,42], it is expected that a similar phenomenon may occur in Ghana. Thus the continuous monitoring of the prevalence of these mutations in parasites in the country is important. Such information is crucial for consideration of chloroquine as prophylaxis for malaria risk groups like children and pregnant women. With the use of ACT in the country, *pfmdr1* copy number determination will help in the early detection of artemisinin derivatives resistance which has implications for chemoprophylactic drug recommendations for natives and travelers from non-endemic regions. This study investigated the prevalence of mutations in *pfcrt* and *pfmdr1* genes as well as the gene copy number of *pfmdr1* in clinical isolates collected from nine sentinel sites in Ghana to detect the trends in the prevalence over seven years.

Methods
Study sites
Samples used in this investigation were collected from 2003–2010 from nine sentinel sites in Ghana established for monitoring anti-malarial drug resistance in the country. These sites are located in the three ecological zones of Ghana: Begoro, Bekwai, Hohoe, Sunyani and Tarkwa are located in the tropical forest ecological zone and experience perennial malaria transmission. Navrongo, Wa and Yendi are in the Guinea Savanna ecological zone and experience seasonal malaria transmission. Cape-Coast is in the Coastal Savanna ecological zone and experience perennial malaria transmission.

Study samples
Blood samples were collected from children aged six to 59 months presenting with uncomplicated malaria at the sentinel sites after the parents or guardians of these children gave informed consent for the children to participate in the study [43-45]. Filter-paper blood blots were made for each child, air-dried and stored in zip-locked bags with silica gel at room temperature until use.

Determination of *pfmdr1* gene copy number
In all, 756 filter paper, blood-blot samples collected from 2003–2010 were used for the *pfmdr*1 copy number (CN) determination. The real-time quantitative PCR (qRT-PCR) method was used following a published protocol [36]. Parasite DNA was extracted using the QIAamp DNA Blood Mini Kit. The *pfmdr1* gene copy number was estimated for all the parasite isolates by the relative ΔΔCt method [36]. The DNA from *P. falciparum* cell lines 3D7, K1, W2mef and DD2 with copy numbers of 1, 1, 2 and 3–4, respectively were used as references. Samples were

run in triplicates for the quantitative PCR and repeated additional times for samples with estimated CN values above 1.5. The estimated CN values were rounded to the nearest integer. For the selection of monoclonal infections from the entire data, the merozoite surface protein 2 (*msp2*) genotyping analysis was done on all 756 samples for FC27 and IC1 genotypes following published protocols [46,47]. Monoclonal infections were selected samples with only one *msp2* genotype; either FC27 or IC1with one PCR band seen on the electrophoresis gel, which were scored by two people.

Detection of *pfmdr1* and *pfcrt* polymorphisms
In all, 1,102 samples were analysed for the *pfmdr1* polymorphisms at codon 86 (asparagine to tyrosine) and 832 samples for codons 184 (tyrosine to phenylalanine), 1034 (serine to cysteine), 1042 (asparagine to aspartic acid) and 1246 (aspartic acid to tyrosine). For *pfcrt* polymorphism at codon 76 (lysine to threonine), 1,063 samples were analysed. DNA was extracted from the filter-paper blood blots using the Tris EDTA buffer extraction method [48]. PCR followed by restriction fragment length polymorphism (RFLP) was used to detect the mutations using published protocols for *pfmdr1* [13] and *pfcrt* [5].

Data analysis
The percentage of isolates with increased *pfmdr1* gene copy number was determined for each sentinel site for the time points; 2003–04, 2005–06, 2007–08, and 2010. The data were then pooled for the percentage of Ghanaian isolates with increased copy number and the observed trend determined. The prevalence of mutations for *pfmdr1* gene codons 86, 184, 1034, 1042 and 1246 and *pfcrt* codon 76 were determined for each site and an overall data was pooled from the sites for the Ghanaian isolates analysis. The significance of the observed trends in the prevalence of the alleles over the years from 2003 to 2010 was determined using the Chi-squared test for trends (EpiCalc 2000).

Ethics
Ethical approval for this study was received from the Noguchi Memorial Institute for Medical Research Institutional Review Board (NMIMR IRB) and the United States Naval Medical Research Unit No 3 (NAMRU-3) IRB.

Results
Increased *pfmdr1* gene copy number in Ghanaian isolates
Seven-hundred and fifty-six (756) samples were analysed for the detection of *pfmdr1* gene copy number. Of these, 47 samples (6.3%) were collected in 2003–2004, 171 (22.7%) were collected in 2005–06, 415 (54.9%) collected in 2007–08, and 121 (16%) collected in 2010. From the MSP2 genotyping analysis done, 53.2% (25/47) of the

samples from 2003–04 had either FC27 allele (one PCR band seen) or IC1 allele (one PCR band seen) and, therefore, were termed as monoclonal infections. For the 2005–06, 2007–08 and 2010, 48% (82/171), 63.4% (263/415) and 62.8% (76/121), respectively, had monoclonal infections. The total number of samples with monoclonal infections (as defined with MSP2 genotyping) used in the data analysis from 2003–2010 was 446 (59%) out of the 756 samples analysed.

The number of experimental repeats, range and mean ± standard deviation of the estimated copy number (CN) for the *P. falciparum* clones 3D7, K1, W2-mef and Dd2 are shown in Table 1. The samples with estimated CN values above 1.5 were repeated for confirmation. The number of isolates with increased *pfmdr*1 gene copy number from the nine sites per year is shown in Table 2. The sentinel sites with the majority of increased gene copy number per year were Navrongo (4%, 1/25) for 2003–04, Begoro (10%, 8/82) for 2005–06, Yendi (2.3%, 6/263) for 2007–8, and for 2010, Tarkwa, Navrongo and Wa (each with 4%, 3/76). Estimated CN of isolates from monoclonal infections for all the sites per year are shown in Figure 1. The highest estimated CN was 2.5 and the lowest was 0.6 for the isolates with monoclonal infections.

Decline in the prevalence of *pfcrt* T76 and *pfmdr*1 Y86 alleles after change in drug policy

In all, 1,063 samples were analysed for the detection of the *pfcrt* codon 76 alleles (K or T) using PCR followed by RFLP. Of this number of isolates, 324 (30.5%) were collected in 2003–04, 232 (21.8%) collected in 2005–06, 402 (37.8%) collected in 2007–08, and 105 (9.9%) were collected in 2010 from the sentinel sites. The prevalence of the T76 (chloroquine resistance allele) as observed from the sentinel sites ranged from 50-98% in 2003–04, 73-95% in 2005–06, 50-95% in 2007–08 and 45-80% in 2010. Whilst for K76 (sensitive allele), the ranges of the prevalence from the sites were 4-52% in 2003–04, 5-30% in 2005–06, 6-62% in 2007, and 25-76% in 2010. With regard to the isolates with both alleles of *pfcrt* codon 76 (K76T), the observed prevalence when the data were pooled for Ghana are 2, 10% 12, and 20% from 2003–04, 2005–06, 2007–07 and 2010, respectively. The observed

Table 1 Estimated *pfmdr1* gene copy number of laboratory strains used as controls

	pfmdr1 CN	No. of RT-PCR runs	Range of estimated CN	Mean estimated CN ± SD
3D7	1	30	0.76 - 1.40	1.11 ± 0.15
K1	1	30	0.79 - 1.41	1.16 ± 0.16
W2mef	2	4	1.86 - 2.34	2.11 ± 0.22
DD2	3-4	4	3.43 - 4.06	3.77 ± 0.34

Table 2 Percentage of isolates with increased *pfmdr1* gene copy number in monoclonal infections for each site per year

Sites	%CN> 1			
	2003-04	2005-06	2007-08	2010
Begoro	-	42 (8/19)	0 (0/38)	0 (0/9)
Bekwai	-	0 (0/5)	10 (2/22)	20 (2/10)
Cape-Coast	-	55 (6/11)	3 (1/32)	7 (1/14)
Hohoe	0 (0/12)	-	7 (2/29)	-
Navrongo	8 (1/13)	20 (3/15)	3 (1/29)	30 (3/10)
Sunyani	-	12 (1/8)	10 (3/30)	0 (0/10)
Tarkwa	-	0 (0/2)	27 (4/15)	100 (3/3)
Wa	-	0 (0/2)	12 (4/33)	33 (3/9)
Yendi	-	20 (4/20)	17 (6/35)	18 (2/11)
Total	**4 (1/25)**	**27 (22/82)**	**9 (23/263)**	**18 (14/76)**

trend of the prevalence of the alleles over the years before and after the change in drug policy is shown in Figure 2A. The prevalence of the *pfcrt* 76 alleles for all the sites is shown in Additional file 1. A significant decreasing trend in T76 ($x^2 = 43.49$, p < 0.001) and an increasing trend in K76 ($x^2 = 64.50$, p < 0.001) were observed from 2003 to 2010 (Figure 2A).

The *pfmdr1* codon 86 alleles (N or Y) were detected in 1,102 samples. Of this, 324 (29.4%) samples were collected in 2003–04, 272 (24.6%) were collected in 2005–06, 397 (36%) collected in 2007–08, and 109 (9.9%) in 2010 from the sentinel sites. The prevalence of the resistant allele Y86 for all the individual sites ranged from 48-96% in 2003–04, 31-67% in 2005–06, 36-67% in 2007–08, and 10- 50% in 2010. For the chloroquine-sensitive allele N86, the prevalence ranged from 34-71% in 2003–04, 65-89% in 2005–06, 71-100% in 2007–08, and 80-100% in 2010. A general trend of increase in the prevalence was observed for N86 ($x^2 = 96.31$, p < 0.001) and a decrease in Y86 ($x^2 = 38.52$, p < 0.001) alleles from 2003 to 2010 (Figure 2B).

Prevalence of isolates with *pfmdr1* codons 184, 1034, 1042 and 1246 alleles

Pfmdr1 codons 184 (Y and F), 1034 (S and C), 1042 (D and N) and 1246 (D and Y) alleles were detected in 832 samples. Out of this total number, 54 (6.5%) samples were collected in 2003–04, 272 (32.7%) were collected in 2005–06, 397 (47.7%) were collected in 2007–08, and 109 (13.1%) collected in 2010. The ranges of prevalence of the *pfmdr1* Y184 for all the sites per year are 22-74% for 2003–04, 18-65% for 2005–06, 30-53% for 2007–08, and 20-60% for 2010. For the *pfmdr1* F184 mutant allele, the ranges were 26-78%, 35-82%, 48-70%, and 40-80% for 2003–04, 2005–06, 2007–08, and 2010, respectively. The prevalence of the
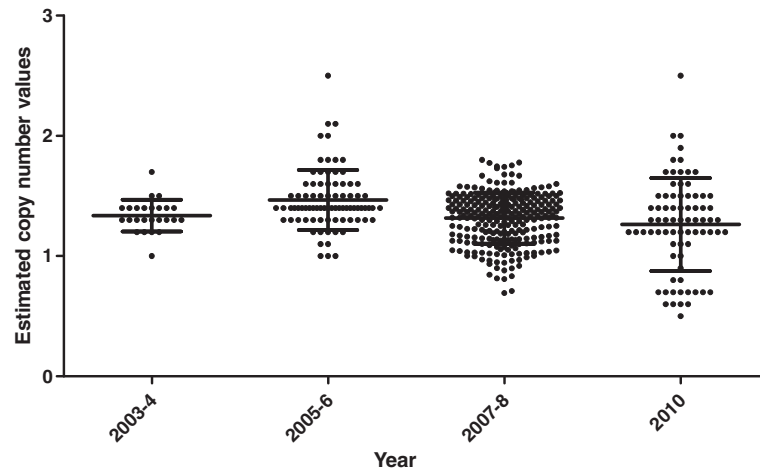
Figure 1 Estimated copy number values for parasite isolates from Ghana per time points. Each dot represents the estimated copy number value per parasite. Lines represent the mean and standard deviation of the estimated copy number value for the isolates.
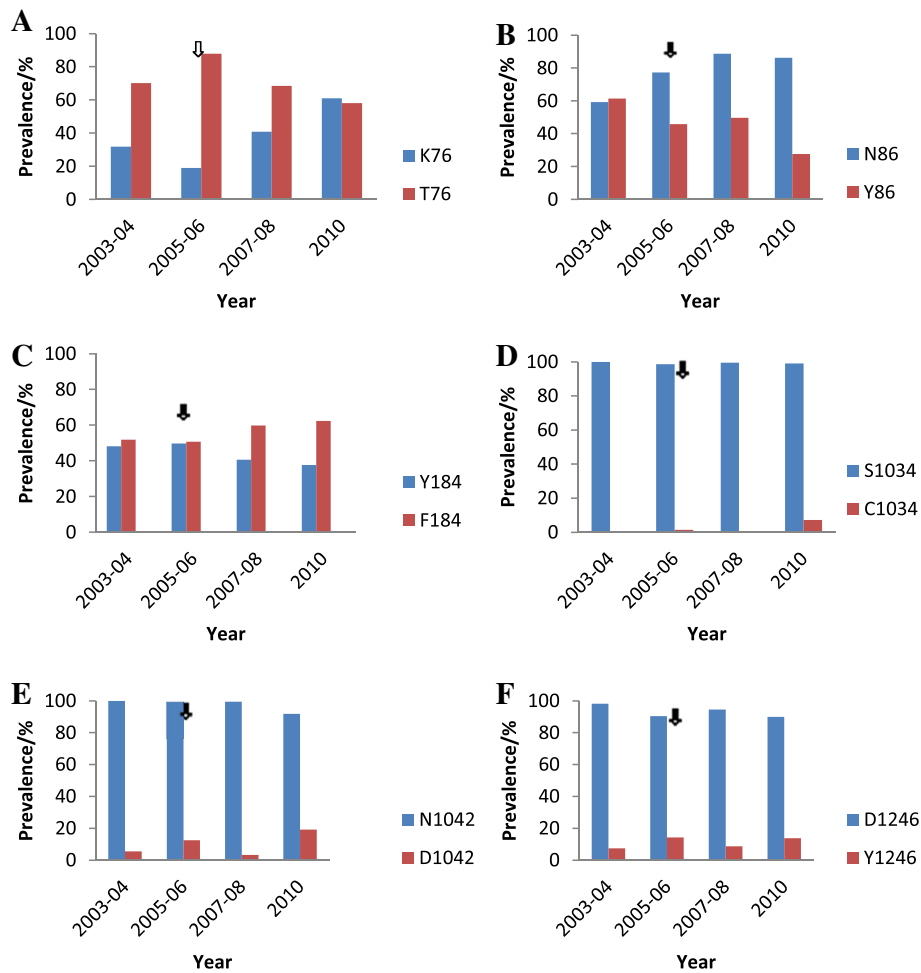

Figure 2 Trends in the prevalence of *pfcrt* codon 76 and *pfmdr1* codons 86, 184, 1034, 1042 and 1246 alleles prior to and after the change in treatment policy. The arrow indicates the period of anti-malarial drug policy change. **(A)** *pfcrt* 76, **(B)** *pfmdr1* 86, **(C)** *pfmdr1* 184, **(D)** *pfmdr1* 1034, **(E)** *pfmdr1* 1042 and **(F)** *pfmdr1* 1246.

pfmdr1 codon 184 alleles for all the sites per year is shown Additional file 1. The observed increasing trend of F184 and the decreasing trend in the prevalence of Y184 alleles did not reach statistical significance ($x^2 = 7.39$, p = 0.060; $x^2 = 7.49$, p = 0.057, respectively) (Figure 2C).

The wild type allele of *pfmdr1* 1034 (S1034) was predominant in the isolates from all the time points. The prevalence of the isolates with the allele was 100% for 2003–04, ranged from 92-100%, 98-100%, and 95-100% for 2005–06, 2007–08 and 2010, respectively. The highest prevalence of the mutant C1034 was 35% and was seen in isolates from Wa (2010). The prevalence of the *pfmdr1* codon 1034 alleles for all the sites per year is shown in Additional file 1. The prevalence of the alleles was stable through the time points (Figure 2D).
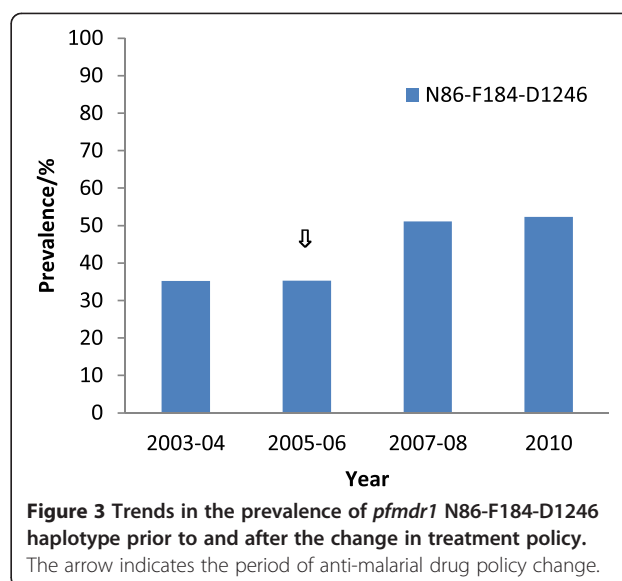
The *pfmdr1* N1042 allele, which is the wild type allele, was present in majority of the isolates such that all the samples from 2003–04 had the allele (100%). The prevalence of the allele for 2005–06, 2007–08 and 2010 ranged from 97-100%, 98-100% and 65-100%, respectively. For the mutant allele, D1042, the highest prevalence of 73% was observed in isolates from Begoro (2010). The prevalence of the *pfmdr1* codon 1042 alleles for all the sites per year is shown in Additional file 1. There was stability in the prevalence of the *pfmdr1* codon 1042 alleles over the years (Figure 2E).

The codon 1246 wild type allele was present as the majority allele in the isolates for all the time points. The prevalence ranged for 2003–04, 2005–06, 2007–08 and 2010 from 96-100%, 80-100%, 94-100%, and 75-100%, respectively. For the mutant allele, Y1246, the highest prevalence was 35%, which was observed in the isolates from Cape-Coast (2010). The prevalence of the *pfmdr1* codon 1246 alleles for all the sites per year is shown in Additional file 1. The prevalence of the alleles was stable over the years (Figure 2F).

The prevalence of isolates with the *pfmdr1* N86-F184-D1246 (NFD) haplotype showed a significant increasing trend ($x^2 = 20.75$, p <0.001) (Figure 3). For the Y86-F184-D1246 haplotype, the prevalence observed for 2003–04, 2005–06, 2007–08, and 2010 was 31.5, 25.0, 31.0 and 18.3%, respectively. Whilst for the Y86-Y184-Y1246 haplotype, the prevalence was 4.0, 6.0, 5.0, and 5.0% for 2003–04, 2005–06, 2007–08, and 2010, respectively. The *pfmdr1* wild type N86-Y184-S1034-N1042-D1246 haplotype was present in 35.2, 33.5, 32.5, and 26.6% of the isolates, respectively for 2003–04, 2005–06, 2007–08, and 2010.

Discussion

In the absence of any reported parasite resistance to ACT in Ghana, the increased *pfmdr1* gene copy number observed in this study indicates the possibility of nascent clones of *P. falciparum* with reduced susceptibility to the artemisinin derivatives in the country. The high frequency

Figure 3 Trends in the prevalence of *pfmdr1* N86-F184-D1246 haplotype prior to and after the change in treatment policy. The arrow indicates the period of anti-malarial drug policy change.

of malaria in Ghana, coupled with a solitary reliance on ACT treatment, poses a situation where selection and propagation of drug-resistant lineages is highly possible. The malaria treatment policy was changed in 2005 from the use of chloroquine as first-line drug to the use of AS-AQ, and in 2008, AL and DHAP were added as the first-line drugs for the treatment of uncomplicated malaria in Ghana. The data showed that before the change, 2003–04, only one parasite out of the 25 isolates tested had increased copy number of 2. This outcome, which is expected, reflects the absence of the artemisinin derivatives and mefloquine drug pressure as these drugs were in limited use at that time. However, it is interesting to note that soon after the implementation of the new malaria drug policy in 2005–06, 27% of the isolates showed an increased *pfmdr1* gene copy number, which was the highest of the four time points. This observation can be attributed to the drug pressure induced by the increased use of the artesunate, a component in the ACT.

Although there is no evidence data on the use of artesunate in the country before 2005, due to the low sensitivity of parasites to chloroquine then, there was limited use of the drug as monotherapy for the treatment of uncomplicated malaria (Neils B. Quashie, pers comm). After 2005–06 there was a decline from 27% in 2005–6 to 9% in 2007–08 with the introduction of the other two ACT, AL and DHAP. There was an increase from 9% to 18% of isolates with copy number increase in 2010. The observed trend of the parasites with increased copy number at the designated time points in the surveillance is suggestive that there is circulation of parasites with reduced sensitivity to artemisinin derivatives and mefloquine in Ghana. Of the three ecological zones in Ghana, isolates from the three savanna zone areas had the majority with

increased gene copy number; Navrongo for 2003–04, Yendi for 2007–08, Wa and Navrongo for 2010. It is, therefore, not surprising that Abuaku and others reported a delayed clearance of parasites with the use of AL, especially with parasites from the savannah zone [43]. This finding adds credence to the assertion that gene copy number variation (CNV) may be the suitable indicator for incipient delays in parasite clearance with the use of ACT. CNV in this case involves the duplication of multi-drug resistance genes which help the parasite to survive under unfavourable drug conditions and as such the *pfmdr1* increased copy number is alleged to confer resistance to some ACT and a decrease in the gene copy numbers may confer sensitivity to some anti-malarials [49-51].

The increased *pfmdr1* gene copy number being recorded in sub-Saharan Africa can be worrying although it is not a good indicator of ACT sensitivity in SE Asia it is important to monitor the prevalence of this marker in the circulating parasite population. With ACT use and vector control, the expectation of reduction in mortality and morbidity in African children will be dwindled with the emergence and spread of resistant parasites to the newly introduced ACT. Since the first report of ACT resistance in Southeast Asia and the associated *pfmdr1* gene copy number, the gradual movement of these parasites into Africa is being seen. Increased *pfmdr1* gene copy has been reported in West African countries such as Côte d'Ivoire, Burkina Faso, Togo [52], Senegal [30], but not in Nigeria [23], the Gambia (David J Conway, pers comm), Liberia and Guinea Bissau [53]. Other African countries with reported increased *pfmdr1* copy number in isolates includes Kenya [9], Gabon [31] and Sudan [54] but not in Cameroon [27] and Mozambique [55].

Chloroquine was the first-line drug for the treatment of uncomplicated malaria in Ghana before the change to the use of ACT. In 2007 Koram's group reported the association between the molecular markers of chloroquine resistance, *pfcrt* T76 and *pfmdr1* Y86 with drug treatment failure in a clinical study [16]. This report [16] together with that from *in vivo* [45] and *in vitro* [56] studies ran in parallel, which was made available to the National Malaria Control Programme (NMCP), became one of the basis for treatment policy change in the country. Since then, there has been continuous monitoring of anti-malarial drug resistance in the country using the three aspects of investigation named above. An increasing trend in the prevalence of the *pfcrt* K76 wild type allele from 19 to 59% from 2005 to 2010 and a decreasing trend for *pfcrt* T76 from 88 to 56% for the same period were observed. The general observation depicts a decline of *pfcrt* T76 with and a gradual takeover by K76 after chloroquine was removed in 2005 as the first choice of anti-malarial treatment. This observed trend in Ghana is consistent with observations of decreasing trend of the

mutants in Malawi, where the decrease was from 85 to 13% for 1993 to 2000 [41], Kenya, a decrease from 95 to 60% for 1993 to 2006 [42], Senegal, a decrease from 92 to 37% for 2000 to 2010 [30] and Mozambique, from 96 to 32% for 2006 to 2010 [55]. The significant decrease in the prevalence of *pfcrt* T76 in Ghana is indicative of the fitness, which is low for the resistant allele after the selective drug pressure has been withdrawn. This same scenario was observed for *pfmdr1* N86Y, where there was a decline in the prevalence of the mutant from 46 to 28% for 2005 to 2010 and an increase in the prevalence of the wild type strains N86 from 77 to 86%. Similar observations have been made in other studies conducted in Africa where K76T and N86Y were investigated concurrently [30,41,55]. In this study, there was no significant difference between the prevalence of N86 for 2007–08 and 2010, which were 89 and 86%, respectively. This is indicative of a gradual gaining of stability of these genotypes in the population similar to what was observed by Nzila's group in Kenya [42].

Despite the comparatively faster decline of T76 in the population, the Y86 seems to reduce slowly in this investigation. A study conducted in Gabon with isolates collected from 2004 and 2009, showed stability in T76 with 94% in 2004 and 96% in 2009 whilst the Y86 increased from 16 to 31% for 2004 and 2009 [57]. The apparent disappearance of the chloroquine-resistant parasites with *pfcrt* T76 and *pfmdr1* Y86 from most of the sub-Saharan Africa malarious areas will help in the re-introduction of the cheapest anti-malarial in combination with an artemisinin derivative. The presence of the mutant parasites with *pfcrt* T76 and *pfmdr1* Y86 is a threat to some of the ACT in use. It is important to note that the prevalence of T76 in 2003–04 was 70% and in the year of implementation of AS-AQ in 2005, the prevalence went up to 88%, which is suggestive that another aminoquinoline, in this case, AQ selected the *pfcrt* codon 76 mutants. This is similar to observations made in studies conducted in Uganda and Burkina Faso [11,28,32]. A report from an *in vitro* study conducted in Nigeria showed an association between the T76 mutation and decreased susceptibility to artemether [58]. The increasing trend in the prevalence of N86 observed in this study must be closely monitored since this allele is reportedly linked to decreased sensitivity of parasites to lumefantrine and mefloquine and it is also a marker for lumefantrine tolerance [57]. In addition an increasing trend for K76 will create a future problem for ACT use because it has been seen in recrudescent samples after AL use [59].

The prevalence of the other SNPs of the *pfmdr1* gene was also investigated, which included the alleles of codons 184, 1034, 1042 and 1246. The mutant allele of codon 184 is known to be selected after AL use in recrudescent samples [54,60]. From the results, in 2003–04,

the prevalence was about 52% and it reduced to 51% in 2005 and then increased to 62% in 2010. Although the increasing trend of the prevalence of F184 from 2003 to 2010 was not significant, there is an indication of selection of this suggested AL resistance allele. Results from a 2010 study conducted in seven communities in southern Ghana revealed the prevalence of F184 to be about 72% [61]. In the West African region, a report from a study conducted in Senegal also showed the prevalence of 68% in samples collected in 2009–2010 [30]. The wild type allele, Y184 was observed to be decreasing in prevalence whilst the other alleles, such as the S1034 wild type allele, were stable over the time points. An increase in the prevalence of the mutant (C1034) was observed for 2010. For the codon 1042 alleles, the wild type N1042 was also stable until a decrease in prevalence (from 100 to 92%) was observed in 2010. The mutant D1042 was also about 19% in 2010 with low to non-existent levels in the previous years. For the codon 1246 alleles, D1246 dropped from 98% in 2003–04 to 90% in 2010 whilst the Y1246 was observed to be gradually increasing 7% in 2003–04 to 14% in 2010. The Y1246 mutant has been linked to quinine failure [57], as such the use of quinine as second-line drug in Ghana for the treatment of uncomplicated malaria may delay the selection of these mutant parasites. There is more stability in the three *pfmdr1* codons 1034, 1042 and 1246 compared to the 86 and 184. The haplotype mutant of *pfmdr1* C1034-D1042-Y1246 has been linked to increased sensitivity to artemisinin [34,35] whilst C1034-D1042 is linked to sensitivity to mefloquine [30]. Furthermore, the Y86-F184-D1042 mutations have been shown to increase the susceptibility of parasites to aryl-amino-alcohol drugs, artemisinin derivatives, mefloquine, halofantrine and lumefantrine [28-32]. As such the stability of these mutants as observed in this study will help maintain the use of ACT in the country. The N86-F184-D1246 haplotype, which was detected in 35% of the isolates in 2003–04, increased significantly to 53% in 2010. Similar trends in prevalence for the NFD haplotype have been reported in Tanzania and Mozambique after the introduction of AL use [24-26]. In addition, this haplotype as well as *pfcrt* K76 were seen in recrudescent samples after AL use [54,59]. The NFD haplotype therefore needs to be monitored effectively especially in malarious countries using AL for chemotherapy.

Conclusions

Generally, the observations made in this study with the prevalence of the molecular markers are in line with what is expected before and after the change of the malaria treatment policy. The increase in *pfmdr1* gene copy number (although not a good indicator) in some of the isolates, which is linked to susceptibility of artemisinin

derivatives, mefloquine, halofantrine and lumefantrine, has implications for ACT use in Ghana. As the chloroquine resistant genotypes decrease in frequency, and subsequently *in-vitro* data confirms increased susceptibility to the drug, it can be introduced as prophylaxis for malaria risk groups, such as children and pregnant women. The findings draw attention to the need to continually monitor molecular markers of all the anti-malarial drugs currently in use in Ghana to allow for early detection of reduced or increased parasite susceptibility to the drugs. It must be stressed that findings from this study have given some insight into the genetic background of the parasites in circulation in Ghana. This will enable genuine conjuncture into the future of malaria in the country in the context of the SNPs of anti-malarial drug resistance and the potential effect of the increasing trends of the drug-resistant parasite populations. Further monitoring of these markers are ongoing in Ghana for ACT efficacy, including both *in vivo* and *in vitro* studies.

Additional file

Additional file 1: Prevalence of *Plasmodium falciparum* infections with *pfcrt* and *pfmdr1* alleles at the nine sentinel sites in Ghana for the study time points.

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