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High prevalence of *PfCRT* K76T mutation in *Plasmodium falciparum* isolates in Ghana

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ABSTRACT

Plasmodium falciparum has successfully developed resistance to almost all currently used antimalarials. A single nucleotide polymorphism in the *P. falciparum* chloroquine resistance transporter (*Pfcrt*) gene at position 76 resulting in a change in coding from lysine to threonine (K76T) has been implicated to be the corner stone of chloroquine resistance. Widespread resistance to chloroquine in endemic regions led to its replacement with other antimalarials. In some areas this replacement resulted in a reversion of the mutant T76 allele to the wild-type K76 allele. This study was conducted to determine the prevalence of the K76T mutation of the *Pfcrt* gene eight years after the ban on chloroquine sales and use. A cross-sectional study was conducted in 6 regional hospitals in Ghana. PCR-RFLP was used to analyse samples collected to determine the prevalence of *Pfcrt* K76T mutation. Of the 1318 participants recruited for this study, 246 were found to harbour the *P. falciparum* parasites, of which 60.98% (150/246) showed symptoms for malaria. The prevalence of the *Pfcrt* T76 mutant allele was 58.54% (144/246) and that of the K76 wild-type allele was 41.46% (102/246). No difference of statistical significance was observed in the distribution of the alleles in the symptomatic and asymptomatic participants ($P = 0.632$). No significant association was, again, observed between the alleles and parasite density ($P = 0.314$), as well as between the alleles and Hb levels of the participants ($P = 0.254$). Notwithstanding the decline in the prevalence of the *Pfcrt* T76 mutation since the antimalarial policy change in 2004, the 58.54% prevalence recorded in this study is considered high after eight years of the abolishment of chloroquine usage in Ghana. This is in contrast to findings from other endemic areas where the mutant allele significantly reduced in the population after a reduction chloroquine use.

1. Introduction

Antimalarial drug resistance is a major threat to health in endemic areas. The arsenal of antimalarial drugs is limited and most of these have become obsolete because the parasites have developed resistance to them. *Plasmodium falciparum* has developed resistance to almost all currently used antimalarials – amodiaquine, chloroquine (CQ), mefloquine, quinine and sulfadoxine–pyrimethamine (WHO, 2006) and currently to the artemisinins (Dondorp et al., 2009).

Chloroquine resistance (CQR) in Ghana was first alleged in 1965 (Beausoliel, 1967), however, resistance *P. falciparum* strains were finally isolated in 1987 (Neequaye et al., 1987). Reduced parasite susceptibility to the drug was continuously reported from then (Ofori-Adjei et al., 1988; Afari et al., 1992) till CQ was replaced by Artemisinin-based combination therapy in 2004 (WHO, 2008).

A single nucleotide polymorphism (SNP) in the *Pfcrt* gene at position 76 resulting in a change in coding from lysine to threonine (K76T) has been shown by transfection and epidemiological studies to be the corner stone of CQR (Djimdé et al., 2001; Durand et al., 2001; Sidhu et al., 2002; Lakshmanan et al., 2005) but in and of itself does not confer CQR. Other SNPs of the Pfcrt associated with CQ response are N75E/K, Q271E and R371I (Fidock et al., 2000; Wootton et al., 2002).

In regions where CQR persisted, replacement of CQ with other antimalarials resulted in reversion of the mutant T76 to the wild-type K76 (Kublin et al., 2003; Laufer et al., 2006; Mwai et al., 2009;

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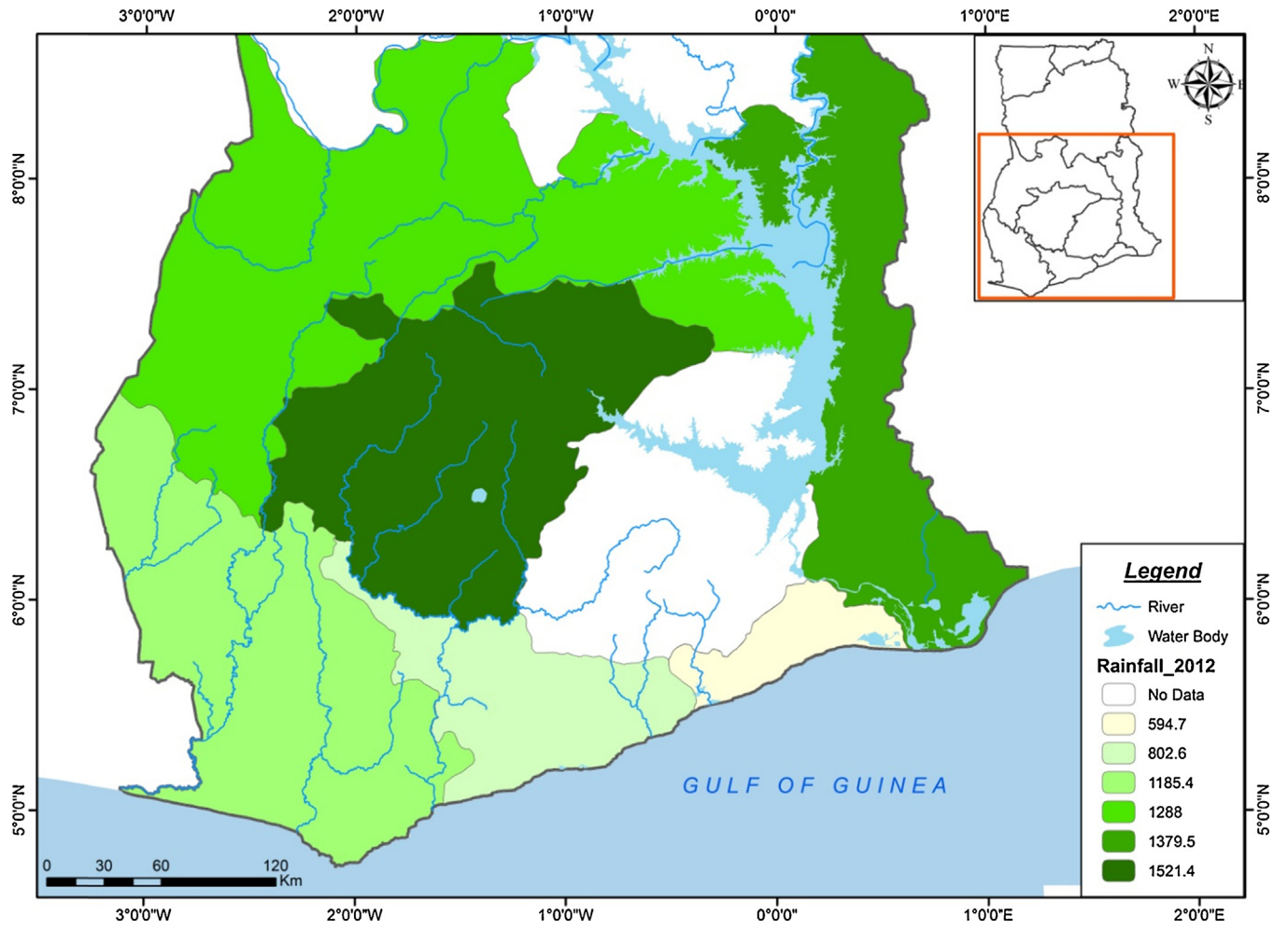

Fig. 1. 2012 annual average rainfall of study sites (rainfall data were supplied by Ghana Meteorological Agency, Accra; Map was designed by the GIS and Remote Sensing Unit of the Department of Geography, University of Cape Coast).

Thomsen et al., 2013), suggesting that CQR may be at a fitness cost to the parasite (White, 2009). While the reversion has been very rapid some countries/regions, it has been very slow in others. Differences in drug policy implementation between countries partly account for this discrepancy (Mohammed et al., 2013).

CQ sales and use have been banned in Ghana following the adoption of the ACT treatment policy. We have, however, recently discovered CQ sales in some drug retail shops and also detected traits of CQ in urine samples of patients visiting health facilities in the Central Region of Ghana (unpublished data).

This study was aimed at determining the prevalence of the K76T mutation of the *Pfcrt* gene eight years after the ban of CQ sales and use in Ghana.

2. Methods

2.1. Study sites and sampling

Samples for this study were collected from regional hospitals in the Ashanti, Brong-Ahafo, Central, Greater Accra, Western and Volta regions of Ghana from April 2010 to May 2011. The regional hospitals serve as referral centres for all district hospitals in each of the regions.

The Greater Accra Region is relatively dry since it falls within the dry coastal equatorial climatic zone with temperatures ranging between 20 °C and 30 °C. Brong Ahafo has two main vegetation types, the moist semi-deciduous forest, mostly in the southern and southeastern parts, and the guinea savannah woodland, which is predominant in the northern and northeastern parts of the region. The region generally has a tropical climate, with high temperatures averaging 23.9 °C. The Western Region lies in the equatorial climatic zone that is characterized by moderate temperatures, ranging from 22 °C at nightfall to 34 °C during the day. The Ashanti Region lies within the wet, semi-equatorial forest zone with an average daily temperature of about 27 °C. The Volta Region has a tropical climate, characterized by moderate temperatures, 21–32 °C for most of the year. Central region lies within the dry equatorial zone and moist semi-equatorial zone. It can be broadly divided into two ecological zones; the coast, which consists of undulating plains with isolated hills and cliffs characterized by sandy beaches and marsh in certain areas; and the hinterland, where the land rises between 250 m and 300 m above sea level. Annual average rainfall of these regions is as indicated in Fig. 1.

Malaria is hyperendemic in all the six study sites with seasonal peaks during the rainy seasons. Participants for the study were randomly selected from patients visiting the out-patient department of the regional hospitals. Both symptomatic and asymptomatic patients were recruited into the study.

2.2. Ethical considerations

The study was approved by the Ghana Health Service Ethics Committee (GHS-ERC-16/7/09). Approval was also sought from the medical directors and administrators of the various health

Table 1
Oligonucleotide sequence and PCR conditions used to detect *Pfcrt* K76T SNP.

Primer	Sequence (5′–3′)	Size (bp)	PCR Condition
Primary amplification			
CRT1F	TTGTCGACCTTAACAGATGGCTCAC	526	96 °C, 15′ followed by 40× (96 °C, 30″; 56 °C, 90″; 72 °C, 90″); 72 °C, 10′
CRT1R	AATTTCCCTTTTTATTTCCAAATAAGGA		
Nested amplification			
CRT2F	CTTGTCTTGGTAAATGTGCTC	200	96 °C, 15′ followed by 40× (96 °C, 30″; 50 °C, 90″; 72 °C, 90″); 72 °C, 10′
CRT2R	GAACATAATCATACAAATAAAGT		

facilities before sample collection. The study was explained to the prospective participants in their own language. After ensuring that inclusion criteria were met, written informed consent of the participants or parents/guardians/representatives were sought before sample collection. Anonymity of study subjects was strictly enforced. Samples were collected and handled solely by trained laboratory technologists. The study posed no risk to participants except for the transient pain they felt during blood collection. Sterile techniques and disposable, single use materials were used at all times.

2.3. Participant recruitment

Participants were randomly recruited into the study from the out-patient departments of the selected regional hospitals irrespective of the complaint with which they reported to the hospital. A patient was excluded from the study if he/she was younger than 6 months old, unconscious, haemophilic, or experiencing palpitation at the time of sample collection.

2.4. Sample collection

Five millilitres (5 ml) of blood sample was collected from each participant into tubes containing EDTA by trained and licensed medical laboratory technologists from the regional hospitals. All blood samples collected were stored on ice and transported to the parasitology research laboratory of the Department of Biomedical and Forensic Sciences, University of Cape Coast. About 1 ml of each blood sample collected was spotted on a 3MM Whatman filter paper. The remaining 4 ml of blood collected from each participant was used for other laboratory analyses which are not reported here. The blood spots were air-dried and stored at −20 °C in plastic envelopes containing silica gel.

2.5. DNA extraction

DNA was extracted from all filter paper spots using the described chelex extraction method (Bereczky et al., 2005) with modification. Briefly, about 6 mm × 10 mm portion of each blood spot was incubated overnight at room temperature in 0.5% phosphate buffered saline (PBS). The filter paper punches were then washed twice with 1 ml 1× PBS, transferred into new tubes containing 150 μl of sterile double distilled water and 50 μl of 20% chelex-100, and vortexed for 30 s. The mixture was heated at 95 °C for 10 min, vortexing every 2 min, followed by centrifugation for 5 min at 13,000 rpm. About 140 μl of the supernatants was then transferred into new 1.5 ml tubes, centrifuged for 10 min at 13,000 rpm and 120 μl of the supernatant transferred into new 1.5 ml tubes, to ensure complete removal of chelex beads. All extracted DNA were electrophoretically analysed in horizontal plate with 2% agarose gels, containing 0.1 μg/ml ethidium bromide, and analysed under UV light (Syngene UGenius gel documentation) to check the quality of eluted DNA. Samples which showed trace of DNA as well as those which did not were both included in the PCR-RFLP analysis. The remaining DNA samples were stored at −20 °C.

2.6. PCR-RFLP of Pfcrt

In order to analyse the K76T SNP in the *Pfcrt* gene, a 200 bp fragment was amplified in a nested PCR process. The PCR process was carried out using previously published oligonucleotides (Beck and Ley, 2008) (Table 1). All PCR reactions were targeted for a final volume of 25 μl containing 1× PCR buffer, 3 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphospate (dNTP), 0.2 μM of each oligonucleotide, and 0.05 U/μl of Taq polymerase. PCR amplification was done using Biorad C1000 thermal cycler.

ApoI, acquired from New England Biolabs Inc., Bervely, MA, USA, was used to digest the PCR products. PCR products were used without purification for the restriction, as this will represent the most typical situation in malaria settings (Veiga et al., 2006). The digestion reaction was set at a final volume of 22 μl, comprising 5 μl PCR product, 1 U ApoI and 1× NEBuffer 3. Reactions were then incubated at 50 °C for 6 h and subsequently the ApoI heat inactivated at 80 °C for 20 min. Digested products were electrophoretically analysed in horizontal plate with 2% agarose gel containing 0.1 μg/ml ethidium bromide and analysed under UV light (Syngene UGenius gel documentation).

2.7. Data analysis

Data were entered and validated using Microsoft Office Excel® 2007 (Microsoft Corporation) and analysed with Minitab® Statistical Software version 16 (Minitab Inc.). The count of samples with mutant and wild-type alleles was used to generate the prevalence of the alleles. Student's *t*-test was used to compare the prevalence of alleles in the six study sites and Pearson chi-square test was used to determine the association between the alleles and malaria symptomatology.

3. Results

A total of 1318 participants were successfully recruited for this study; 217 from Greater Accra, 211 from Central, 208 from Western, 206 from Volta, 235 from Ashanti and 241 from Brong-Ahafo regions. The mean age of participants was 32.6 years. Two hundred and forty-six (246) of the participants were found to harbour the *P.*

Table 2
Distribution of *Pfcrt* alleles.

Alleles	G. Accra (*n* = 30)	Central (*n* = 40)	Western (*n* = 34)	Volta (*n* = 32)	Ashanti (*n* = 53)	Brong-Ahafo (*n* = 57)
K76	15	16	13	13	25	20
T76	15	24	21	19	28	36

Table 3
Association between *Pfcrt* K76T mutation and malaria symptom.

	Allele		*P*-value
	K76	T76	
Symptomatic	64	86	0.632
Asymptomatic	38	58	

falciparum parasite; 30 from the Greater Accra, 40 from Central, 34 from Western, 32 from Volta, 53 from Ashanti and 57 from Brong-Ahafo regions. Mean parasite density of these 246 participants was 68,605 parasites/μl. Of the 246 participants, 150 showed symptoms for malaria whereas the remaining 96 were asymptomatic. The prevalence of the *Pfcrt* T76 mutant allele was 58.54% (144/246) and that of the K76 wild-type allele was 41.46% (102/246). The distribution of the two alleles from the six study sites is as shown in Table 2. 57.3% of the 150 symptomatic participants had the mutant T76 allele where as 60.4% of the asymptomatic participants had the mutant allele. No difference of statistical significance was, however, observed in the distribution of the alleles in the symptomatic and asymptomatic participants ($P = 0.632$; Table 3).

Participants with the T76 allele generally had higher parasite densities with a mean parasite density of 73,529 parasites/μl while the mean parasite density for participants with the K76 allele was 61,732 parasites/μl. No significant association was, however, observed between the alleles and parasite density ($P = 0.314$).

No significant association was, again, found between the K76T alleles and anaemia in the participants. The mean haemoglobin levels of the 102 and 144 participants with the K76 and T76 mutations were 10.73 g/dl and 11.09 g/dl, respectively ($P = 0.254$). Again, only 12.75% (13/102) of the participants with the wild-type K76 allele were found to be anaemic and 9.76% (11/144) of the participants with the mutant T76 allele were found to be anaemic ($\chi^2 = 1.768$; $P = 0.184$).

4. Discussion

Prior to the treatment policy change from CQ to Artesunate + Amodiaquine in Ghana, a 64.53% prevalence of the mutant *Pfcrt* T76 allele corresponding to treatment failure with CQ was reported (Duah et al., 2007). From 2005 to 2006, after the replacement of CQ with AS + AQ, the prevalence of the mutation sharply rose to >80% and subsequently declined to about 60% in 2010 (Duah et al., 2013). Samples for this current study were collected from April 2010 to May 2011. The 58.54% prevalence of the *Pfcrt* T76 mutation recorded in this study is considered very high after eight years of the abolishment of CQ usage in Ghana. There is, nonetheless, a decline in the prevalence of the mutation from 2004 to 2011. This decline, however, cannot be said to be rapid comparing it to the situation in Tanzania, Malawi and Mozambique. CQ withdrawal in Tanzania resulted in >90% recovery of susceptibility in ten years of withdrawal (Mohammed et al., 2013). In Malawi, a 100% recovery of the *Pfcrt* K76 was achieved in 13 years after the policy change (Kublin et al., 2003; Laufer et al., 2006) whilst in Mozambique *Pfcrt* K76 prevalence rose from <5% to 80% in only 5 years (Thomsen et al., 2013). Per results from the Duah et al. (2013) study and this current study, the recovery of *Pfcrt* K76 from 2003 to 2011 is from ≈30% to 41.46%. Thus, the decline in prevalence of the *Pfcrt* T76 and the concomitant recovery of *Pfcrt* K76 is relatively slow in Ghana.

Sustenance of the *Pfcrt* T76 mutation may be explained by the continuous use of CQ or the introduction of amodiaquine in Artesunate + Amodiaquine combination. CQ use in Ghana might not have ceased after all since CQ has been found on sale in some few drug retail shops in the central region of the country (unpublished data).

Artesunate + Amodiaquine was the first ACT deployed in Ghana. After several reports of adverse reaction to amodiaquine, Artemether + Lumefantrine and Dihydroartmesinin + Piperaquine were later on added in 2008 (Duah et al., 2013). Thus, three ACTs (AS + AQ, AL and DHAP), are being used concurrently as the first line drugs for treating uncomplicated malaria in Ghana. Cross-resistance between CQ and AQ involving the *Pfcrt* T76 mutant allele has been very well documented (Ochong et al., 2003; Dokomajilar et al., 2006; Holmgren et al., 2006). The sharp rise in the prevalence of PfCRT T76 in 2006 coincided with the introduction AQ in AS + AQ. After introducing other ACTs and hence reducing the AQ pressure, the prevalence of the mutation also reduced. This clearly implicates AQ in sustenance of PfCRT T76. With the current high prevalence of the T76 allele, the level of AQ resistance and treatment failures with Artesunate + Amodiaquine in the country should be monitored urgently. The benefit or otherwise of using three ACTs at the same time should also be reviewed.

Some studies have associated the K76T mutation to severe malaria (Wele et al., 2011; Ranjit et al., 2004; Meerman et al., 2005) while other have found no such association (Giha et al., 2006; Mayengue et al., 2007). We found no association between the *Pfcrt* K76T mutation and disease severity.

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