Drug sensitivity of *Plasmodium falciparum* field isolates to selected antimalarial drugs in Ghana using the *in-vitro* DAPI assay

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Research

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Abstract

Background: Malaria continues to be a major health issue globally with nine out of ten cases reported in Africa. Although the current artemisinin derived combination therapies in Ghana are still efficacious against the *Plasmodium falciparum* parasite, compounding evidence of artemisinin and amodiaquine resistance in the African region establish the need for a full, up-to-date understanding and monitoring of antimalarial resistance to provide evidence for planning control strategies.

Methods: The study was cross-sectional and was conducted during the peak transmission seasons of 2015, 2016, and 2017 in two study sites located in different ecological zones of Ghana involving children aged 0.5-14 years presenting with symptomatic uncomplicated *Plasmodium falciparum (Pf)* malaria with parasitaemia greater than 1000 parasites/µl of blood. Using *in vitro* 4-,6-diamidino-2-phenylindole (DAPI) drug sensitivity assays, 328 *Pf* parasites collected were used to investigate susceptibility to five selected antimalarial drugs: chloroquine, amodiaquine, dihydroartemisinin, artesunate and mefloquine.

Results: The geometric mean B ($GMIC_{50}$) of five drugs against the parasites collected from Cape Coast were 9.6, 23.6, 9.1, 3.5 and 8.1nM for chloroquine, amodiaquine, artemisinin, artesunate, and mefloquine respectively in 2015. There was a 2 fold increase in the $GMIC_{50}$ levels of all the drugs against the isolates collected in 2016 as compared to the 2015 data from Cape Coast .The a of the five drugs against the parasites collected from Cape Coast were significantly higher than those isolates collected from Begoro in 2016 and 2017 ($P<0.001$) . The chloroquine resistance ranged between 1.9% and 9.1% among isolates collected from Cape Coast but remained 0% in Begoro over the period. High amodiaquine resistance levels were recorded at both sites whilst that of artesunate resistance ranged between 4 and 10% over the study period.

Conclusions: The study has assessed the antimalarial drug sensitivities of Ghanaian *Pf* isolates collected over 3 consecutive years. The parasites showed variable resistance levels to all the drugs used over the period. The study has demonstrated the continual return of chloroquine-sensitive parasites. The *in vitro* DAPI assay is a useful method for monitoring individual drugs used in combinations in Ghana for the generation of data on their sensitivities over time.

Background

Malaria continues to be a major health issue globally and efforts are being made to either control or eliminate it in most endemic regions of the world. The World Health Organization (WHO) reported an estimated 92% of all malaria cases and 93% of all malaria reported deaths to have occurred in the African region [1]. It was alarming to note that, five countries of which four were from the African region recorded nearly half of all the malaria cases worldwide with Nigeria accounting for nearly a quarter of these cases with the only country outside the African region being India that recorded 4% of all the reported cases.

The exposure of malaria parasites to sub-optimal drug concentrations increases the possibility of malaria drug resistance [2] and it has been suggested to occur simultaneously with the introduction of the drugs [3]. Resistance has emerged to all antimalarial drugs ever prescribed for the treatment of malaria [4]. Chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) resistance were the first to be documented which mainly affected Southeast Asia, Africa, South America, and some Pacific regions. Artemisinin (ARS) and its derivatives are currently the most potent and rapidly acting antimalarials, recommended by world health organization (WHO) as a component of first-line combination therapy for falciparum malaria in all endemic countries [5, 6].

Unfortunately, there have been reports of the emergence of ARS-resistant parasites in Southeast Asia since 2009, the same place CQ and SP resistance originated from and spread to Africa [7, 8]. Even more alarming are the compounding evidence of reduced susceptibility of *Pf* to artesunate (ART) in Cambodia and the Thai-Myanmar border [6, 9]. Increased resistance to amodiaquine (AMD) has also been documented in South America [10], Afghanistan [11]; first recorded in the African region in Tanzania as early as 2004, then in Angola [12] and more recently in Southwestern Nigeria [13]. Also, findings suggest reduced *Pf* susceptibility to artesunate-mefloquine combination therapy in the Cambodia – Thailand border [14,15] and Benin [16]. Besides, resistance to ARS has not been contained and has now spread beyond Southeast Asia [17]. These findings raise great concern particularly for Africa, where the spread of resistant *Pf* strains bring devastating outcomes [18]. Therefore, there is a need for a full, up-to-date understanding and monitoring of antimalarial resistance to provide evidence for planning control strategies. Controlling the spread of antimalarial drug resistance, especially resistance of *Pf* to artemisinin-based combination therapies, is a high priority because drug resistance has often threatened malaria elimination efforts [6].

Current methods recommended by WHO for monitoring antimalarial drug resistance are: *in vivo* therapeutic efficacy testing, *in vitro* studies to measure intrinsic sensitivity of antimalarial drugs and molecular markers to identify genetic mutations associated with antimalarial drug resistance. These methods complement each other to provide timely information for the national malaria control programme-based treatment policy [19]. In Ghana, subsequent to CQ resistance, the Artesunate Amodiaquine (ART-AMD) combination therapy was adopted for the treatment of uncomplicated malaria in 2004. This treatment regimen has proven effective for over 10 years and is still efficacious [20-22]. However, the compounding evidence of ART and AMD resistance warrants the continuous monitoring and evaluation of Malaria Drug Resistance (MDR) especially when the treatment policy has been running for over a decade. Moreover, there has been evidence of increased *Pf* susceptibility to CQ after the removal of drug pressure [23]. It is also important to monitor drug resistance in areas of varying transmission intensity across the country to indicate hotspots of drug resistance for effective control [24].

This study applied the *in vitro* DAPI assay to study isolates from two study sites (different transmission intensity and patterns) with different drugs currently used in Ghana. In addition, these isolates were tested with previously used chloroquine to observe the re-expansion of the field isolates to the drug.

Methods

Study Area and Population

The study was hospital-based and was conducted in two sites in Ghana with different malaria transmission patterns - the Begoro District Hospital in the Eastern region and Ewim polyclinic in the Central region. The Begoro District Hospital is situated in the Fanteakwa District within the Forest ecological zone of Ghana. The District has an annual rainfall of 1,500 – 2000mm with malaria transmission being intense and perennial [20, 25-26]. The Ewim Polyclinic is situated in the Cape Coast Metropolis within the coastal savannah zone of Ghana where transmission is low to moderate and also perennial with an annual rainfall of 750 – 1000 mm [27]. The malaria transmission pattern is similar to what pertains in other parts of Ghana with two seasonal peaks – major and minor. The major season occurs in April – July with the minor occurring in September- November [28]. The study involved children aged 6 months -14 years presenting with a history of fever, mono-infection with *Plasmodium falciparum* parasite density of 1,000 – 250,000/µL blood; and absence of severe malaria. After meeting the inclusion criteria and informed consent obtained from parents, 4ml of blood was aseptically obtained from each study participant of which 400 µl was used for the DAPI drug assays that was performed within one hour after sample collection.

DAPI Drug Assay

Drugs. The antimalarial drugs used in this study included chloroquine (CQ), amodiaquine (AMD), artesunate (ART), mefloquine (MFQ) and dihydro-artemisinin (DHA). Preparation: the stock concentrations of chloroquine were first prepared in sterile distilled water and then DMSO whereas the rest of the drugs were prepared in DMSO. Aliquots of 20µl of drug were added in duplicates to their respective wells in 96-welled black with clear flat bottom tissue culture treated microtiter plates (Corning, USA) at different working concentrations (2 fold serially diluted) ranging from 750 nM to 2.93 (CQ), 250 to 0.98 nM (AMD), 150 to 0.59 nM (ART), 75 to 0.29 nM (DHA), and 500 to 1.95 nM (MFQ).

***In vitro* drug sensitivity assay.** Parasitized blood samples collected from patients enrolled were washed twice with 10ml incomplete medium (without normal human serum). The erythrocytes were resuspended at 2% hematocrit in complete medium containing RPMI 1640, supplemented with 2% normal human serum, 10% Albumax and 10mg/ml Gentamycin. Blood samples with parasitemia higher than 1% were adjusted to 1% with uninfected O^+ erythrocytes. For each sample, aliquots of 180µl of parasite culture were added to the pre-dosed drugs with a negative control and incubated at 37°C for 72 hours in an air tight chamber containing a gas mixture of 5.5% CO_2, 2% O_2 and 92.5% N_2. The remaining parasite cultures were maintained under the same conditions in T25 culture flasks for daily monitoring which included the change of media and preparation of smears from the culture for observing *Pf* growth using a light microscope. After 72 hours, the processed samples in the plates were harvested by preparing smears from a control well in each plate after which the plates were wrapped in aluminum foil and stored in a freezer at -20^{o}C until ready to be read.

DAPI *P. falciparum* growth assay. This procedure was conducted using a protocol adapted by Ndiaye and colleagues [29] for frozen plate assay. The frozen plates were allowed to thaw at room temperature and spun for 30minutes at 4000rpm. The contents of these plates were discarded and padded on tissue to absorb all well content except the *Pf* DNA which settled as pellets in the wells. Aliquots of 100µl of fluorochrome mixture containing 20 mM Tris-HCl (pH 7.5) (MP Biomedicals, LLC), 5 mM EDTA (Sigma-Aldrich), 0.004% Saponin (Sigma Life Science), 0.01% Trition X-100 (Sigma Life Science), and a 1:75,000 final dilution of 5 mg/ml DAPI (Sigma Life Science) were added to each well containing *Pf* DNA using a multi-channel pipette. This was carried out in the dark since the stain is light sensitive. The DAPI-Buffer-*Pf* DNA mixture was re-suspended in each well using a multi-channel pipette after which the plates were wrapped in aluminium foil to protect them from light and left to incubate at room temperature in the dark for 30 minutes. The incubation was followed with spinning for another 30minutes at 4000rpm and well contents were discarded. Aliquots of 200µl of 1×PBS were added per well using a multi-channel pipette with re-suspension. These plates were kept covered with aluminium foil until reading. The fluorescence from the stained parasite DNA in each plate well was measured using Tecan infinite M200PRO (Ex/Em: 358/461nm, bound to DNA).

Statistical Analysis. The drug concentration inhibiting 50% of parasite activity (IC_{50}) for each drug was estimated from the fluorescence data generated using the online tool, ICE estimator 1.2 generated by Le Nagard and colleagues [30]. The IC_{50} values with their 95% confidence intervals (CI) were calculated by using an Emax model available at https://www.antimalarial-icestimator.net, as $RE = 100 - [(100*C\gamma)/(C\gamma + IC\gamma)]$, where γ is a sigmoidicity factor which expresses the steepness of the curve, RE is the relative effect of the drug (in percent, Y-axis), and C is the drug concentration (X-axis). The estimated drug IC_{50}s were compared over a 3-year period between two study sites. The population means were compared by the Kruskal – Wallis test and the mean difference over time were compared by analysis of variance (ANOVA). The proportions of *Pf* isolates with IC_{50} greater than the resistance threshold from literature [23, 42-43] between the two study sites and between different time points were compared for significant differences using the Chi-square test and Fishers exact test. All statistical analyses and figures were conducted using the software R and graphpad prism respectively. Statistical test were assumed significant at $P < 0.05$.

Results

Baseline characteristics

A DAPI-based *ex vivo* assay was used to monitor the drug sensitivity of *Pf* parasite population circulating in two ecological zones in Ghana with different transmission intensities and over a period of 3 years. The validity of this assay was assessed using the *Pf* laboratory strain 3D7 against the selected drugs under field conditions. The IC_{50} values obtained were comparable to those from other studies. The assay successfully tested for over 71% of the parasite isolates collected in 2015, 73% in 2016 and 54% in 2017. By site, the assay success rate ranged between 73.7and 88.5% for Cape Coast with a similar trend

observed in Begoro for all parasites collected in 2016 whereas for those parasites collected in 2017 the success rates ranged between 54 and 88% for both sites (Table 1). The results of the *in vitro* susceptibility testing are shown in Table 2, Figures 1-4 and supplementary table1.

Table 1: Total *P. falciparum* parasites tested for each site by year and their *in vitro* success rates

	2015 Assay Success % (n/N)	2016 Assay Success % (n/N)		2017 Assay Success % (n/N)	
Drug	**Cape Coast**	**Begoro**	**Cape Coast**	**Begoro**	**Cape Coast**
Chloroquine	78.35 (76/97)	88.5 (54/61)	85.3 (52/61)	62.7 (37/59)	88.0 (44/50)
Amodiaquine	84.54 (82/97)	75.4 (46/61)	73.8 (45/61)	54.2 (32/59)	74.0 (37/50)
Dihydro Artemisinin	79.38 (77/97)	78.7 (48/61)	78.7 (48/61)	54.3 (32/59)	86.0 (43/50)
Artesunate	71.13 (69/97)	86.9 (53/61)	88.5 (54/61)	84.8 (50/59)	54.0 (27/50)
Mefloquine	85.42 (82/96)	88.5 (54/61)	85.5 (54/61)	74.1 (43/58)	76.0 (38/50)

Table 2: The susceptibilities of parasites collected from two sites of varying transmission intensities calculated based on the threshold of resistance of the respective drugs tested

Compound		2015 Resistant isolates n (%)	2016 Resistant isolates n (%)		p value	2017 Resistant isolates n (%)		p value
	Thresholds for resistance	Cape Coast	Begoro	Cape Coast		Begoro	Cape Coast	
Chloroquine	100nM	2 (2.6)	0 (0)	1 (1.9)	0.306	0 (0)	4 (9.1)	0.121
Amodiaquine	60nM	29 (35.4)	24 (52.2)	39 (86.7)	<0.0001	8(25.0)	23 (62.2)	0.003
Dihydro-artemisinin	12nM	50 (64.9)	25 (52.1)	42 (87.5)	<0.001	25(78.1)	35(81.4)	<0.001
Artesunate	12nM	4 (5.8)	2 (3.8)	12 (22.2)	0.008	5 (10.0)	13 (48.2)	0.008
Mefloquine	45nM	0 (0)	6 (11.1)	32 (59.3)	<0.0001	31 (72.1)	34(89.5)	0.050

In both 2016 and 2017, $GMIC_{50}$ for each of the drugs tested in Cape Coast was significantly higher than that in Begoro (Figures 2, 3, and supplementary table 1). There were no statistically significant trends in the GMs for CQ, ART, AMD, and DHAP in both Begoro and Cape Coast, however, there was a significant increase in GM IC_{50} for MQ at both sites (Figure 4).

The CQ resistance in Cape Coast, defined as IC_{50} > 100nM, ranged between 1.9% and 9.1% over the 3 year period (3 years in Cape Coast and 2 years in Begoro). There was no observed CQ resistance in Begoro. AMD resistance in Cape Coast set at IC_{50} > 60nM, reduced from 86.7% in 2016 to 62.2% in 2017. Similarly, AMD resistance in Begoro reduced from 52.2% in 2016 to 25% in 2017. DHA resistance set at $GMIC_{50}$ > 12nM increased from 64.9% in 2015 to 81.4% in 2017 in Cape Coast and from 52.1% in 2016 to 78.1% in 2017 in Begoro. ART resistance set at $GMIC_{50}$ > 12nM also increased from 3.8% in 2016 to 10% in 2017 in Begoro and from 5.8% to 48.2% in 2017 in Cape Coast. The 2016 and 2017 isolates tested showed significantly higher resistance in Cape Coast compared to Begoro, for all drugs except CQ (Table 2).

Discussion

The National Malaria Control Programme in 2005 introduced ART-AMD as the first-line drug for the treatment of uncomplicated malaria after years of chloroquine therapy failures in Ghana [22]. Since the introduction of this combination therapy for the treatment of uncomplicated malaria, the country has continued to monitor its efficacy in addition to other artemisinin-based combination therapies (ACTs) using in vitro methods. The strength of this study is the use of the newly developed DAPI DNA stain [31] to study the susceptibility of clinical isolates to the drugs currently in use in the country. This assay is

robust and a very reproducible marker of parasite growth; it has been demonstrated to be very good in monitoring the development of resistance in parasites at field settings [29]. The study therefore aimed at using this method to investigate the efficacy of commonly used antimalarial drugs in Ghana in addition to chloroquine (currently not in use in Ghana) against clinical parasite isolates causing uncomplicated malaria in different transmission zones of Ghana to aid in providing malaria-drug efficacy surveillance data. SYBR GREEN1 method have been used in Ghana in studying the susceptibility of clinical isolates to a panel of antimalarial drugs in Ghana [23] but to the best of our knowledge, this is the first time the DAPI assay is being used in Ghana and its advantage has been described elsewhere [29]. One major limitation of DAPI assay is the fact that it does not work well at a very low DNA content [46]

In this study, CQ was included as one of the test drugs although it is currently not in use in Ghana and interestingly but not surprising, we found a sharp drop of resistance to CQ ranging between 2 and 9% over the three years monitoring at Cape Coast in comparison with a study done in Ghana in 2012 by Quashie and colleagues [23], where they reported 13% of resistance to CQ. We did not record any resistance to CQ from Begoro which has a higher malaria transmission but that was not surprising because it has been shown that in areas of low transmission such as Cape Coast, the development of resistance is slower but at the same time, it takes a longer time for the resistance to disappear as compared to the high transmission areas [32] such as Begoro and therefore seeing remnants of resistance at Cape Coast is not surprising. Again, it is a drastic drop from the national average of resistance of 56% in 2004 [33] to the 9% we saw over the period (2015–2017). Our data supports findings from Ghana and other parts of Africa which have demonstrated the return of Chloroquine-sensitive strains after the withdrawal of chloroquine as the first-line drug for the treatment of malaria [23, 34–37] with a continuous drop in the prevalence of CQ resistance markers since its withdrawal as the first-line drug for the treatment of uncomplicated malaria.

In Ghana, ART-AMD is one of the first-line drugs for the treatment of uncomplicated malaria and also used to treat pregnant women after the first trimester [22]. This justifies the importance of monitoring the possible development of resistance among the parasites circulating in the country especially to the individual drugs that are used for the combination therapies. Setting the threshold for AMD at 60 nM, many of the parasite isolates were found to be resistant at Begoro between 2016 and 2017 with similar trend also occurring at Cape Coast. These observed in vitro resistant levels does not translate to drug failures as we did report on the therapeutic efficacy outcome of over 90% in the same communities [20]. These in vitro observations could therefore be as a result of an increased tolerance of these parasites to AMD taking into account the slow acting nature of AMD [41].

Again, we observed a resistance ranging between 3.7% and 10% among all the parasites collected in 2016 and 2017 in Begoro when the threshold for the other partner drug, artesunate was set at 12 nM. However, a very interesting observation was made with respect to parasite isolates collected from Cape Coast where we observed a steady increase over the study period

In this study we found a very high DHA resistance in all the parasite isolates studied over the period in both study sites (Table 2). The high DHA and ART in-vitro resistance did not significantly reflect in in-vivo therapeutic efficacy outcomes as earlier described [20]. It has been well documented that artemisinin resistance alone does not necessarily lead to treatment failures [38–39].

Mefloquine is not a recommended drug for the treatment of malaria in Ghana but then it is one of the major prophylactic drugs used by visitors to the country, so it was necessary that it was included in the assessment. In Cape Coast, we did not observe resistance among the isolates tested in 2015 but found 59% and 89% in 2016 and 2017 respectively when the cut off for resistance was set at 45 nM. In comparison, resistance levels among the Begoro isolates were lower 11.11% and 78% for 2016 and 2017 respectively. The sharp emergence of resistant isolates in Cape Coast as compared to Begoro is not surprising because Cape Coast is a Metropolitan with a lot of Tourist activities and therefore attracts a lot of non-immune travelers to the place as compared to Begoro with very little or no tourist activities [40].

It is interesting to note that the observed significantly higher geometric IC_{50} values of each of the drugs tested to be higher in Cape Coast as compared to Begoro for each of the years (2016 and 2017) was not surprising because the transmissions intensities differ in these two communities complicated with the differences in both environmental and socioeconomic conditions all of which have an impact on the development of drug resistance. Although data from this study seems to suggest an increased tolerance to most of the drugs tested, it does not suggest high treatment failures to the antimalarials currently in use in Ghana despite the fact that we do not have concrete data to support what has been found elsewhere [20]. Again, in vitro studies alone cannot be used to explain treatment failures as it is very complex and involves other several factors including host genetic factors, poor adherence and exposure to subtherapeutic doses as well as management and administrative issues [44–45]

In conclusion, we have used the in vitro DAPI assay to assess the sensitivities of over 200 parasites isolates collected over 3 consecutive years from 2 sites of different transmission intensities in Ghana to five drugs (single individual drugs). Although we did see higher resistance levels to all the drugs used over the period (based on the cutoff poits from literature), this does not mean high drug treatment failures because these were tested for individual drugs most of which are used in combinations in Ghana except Chloroquine that is currently not in use in Ghana. This study has clearly demonstrated the continual return of chloroquine-sensitive parasites after its withdrawal as the first-line drug for the treatment of uncomplicated malaria over a decade ago, and higher tolerability of parasites to selected drugs used in artemisinin-based combination therapy. It is therefore prudent to use this method to continually monitor these individual drugs in Ghana.

Abbreviations

CQ: Chloroquine, AMD: Amodiaquine , DHA: Dihydro-Artemisinin, ART: Artesunate MFQ Mefloquine , IC_{50}; 50% Inhibitory concentration $GMIC_{50}$: Geometric Mean IC_{50} DAPI: 4-,6-diamidino-2-phenylindole, PBS: Phosphate Buffered Saline, nM: nano Molar, *Pf: Plasmodium falciparum,*

Declarations

Ehtical Approval:

Approval for this study was obtained from the Noguchi Memorial Institute for Medical Research Institutional Review Board (NMIMR IRB No. 056/12-13). The study aims and objectives, benefits and possible risks were explained to all the parents/guardians of the study participants. Written Informed consent was obtained from each participant prior to enrolment and for those above twelve years, child assent was also obtained.

Consent for Publication:

Not applicable

Availability of data and Materials:

All data generated and/or analysed during this study are included in this published article (and its supplementary information file)

Competing Interest:

The authors declear that they have no competing interest

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Authors Contribution:

MFO, BKA, KAK and AG conceived the study. EEK, BAM EKD, SG, MT and EKB collected the samples. EEK, BAM and EKD executed the experiments. MFO, BAM, BKA and AG, performed the statistical analysis. MFO, EEK , BAM, BKA, KAK and AG contributed in writing the manuscript. All authors read and approved the manuscript.

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Figures

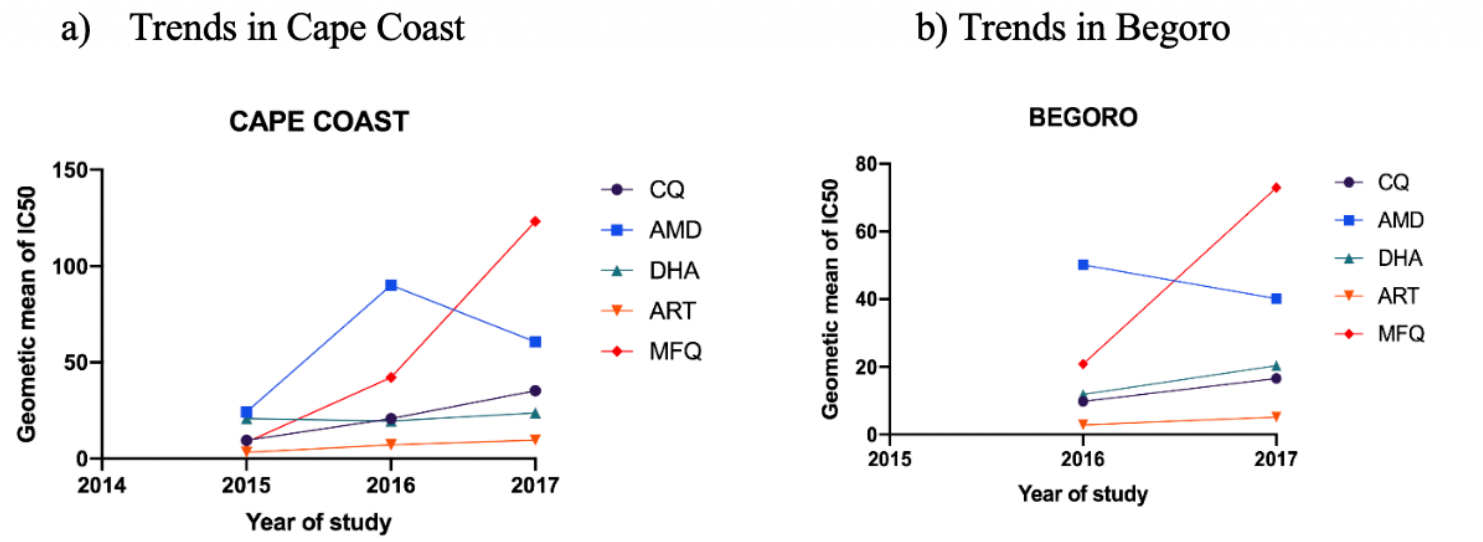

Figure 1

Distribution of IC50 values of Chloroquine (CQ), Amodiaquine (AMD), Dihydro-Artemisinin (DHA), Artesunate (ART) and Mefloquine (MFQ) against P. falciparum parasites collected from Cape Coast in 2015. The black dots represents individual IC50 values and the horizontal lines represent the geometric mean of IC50 and the 95% confidence Intervals (CI)

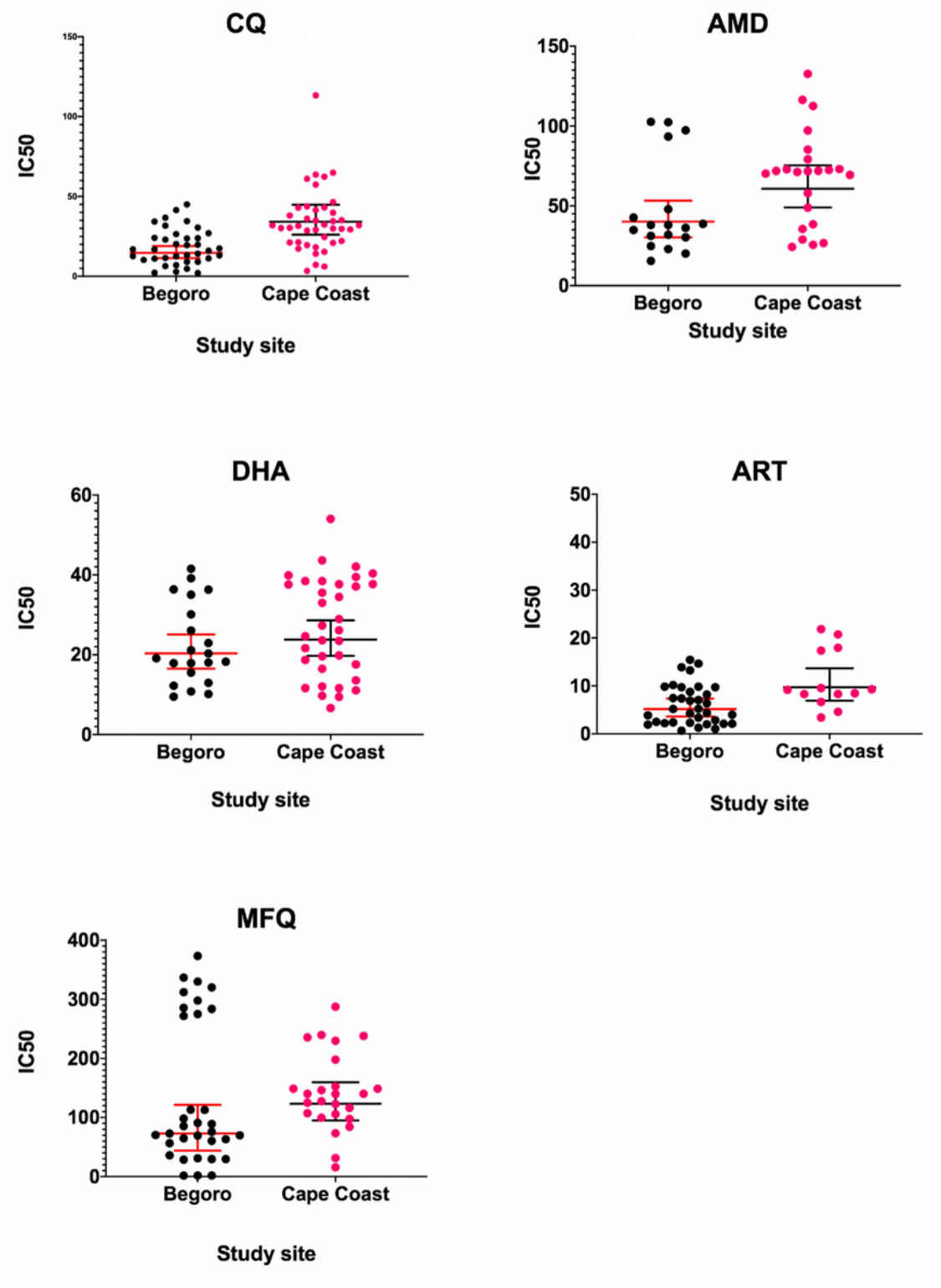

Figure 2

The IC50 values of Chloroquine (CQ), Amodiaquine (AMD), Dihyro-Artemisinin (DHA), Artesunate (ART) and Mefloquine (MFQ) tested against P. falciparum parasites collected from Cape Coast and Begoro in 2016. The black dots represent individual IC50 values of parasites from Cape Coast and the pick dots represents parasites from Begoro. The horizontal lines represent the geometric mean of IC50 with their corresponding 95% CI.

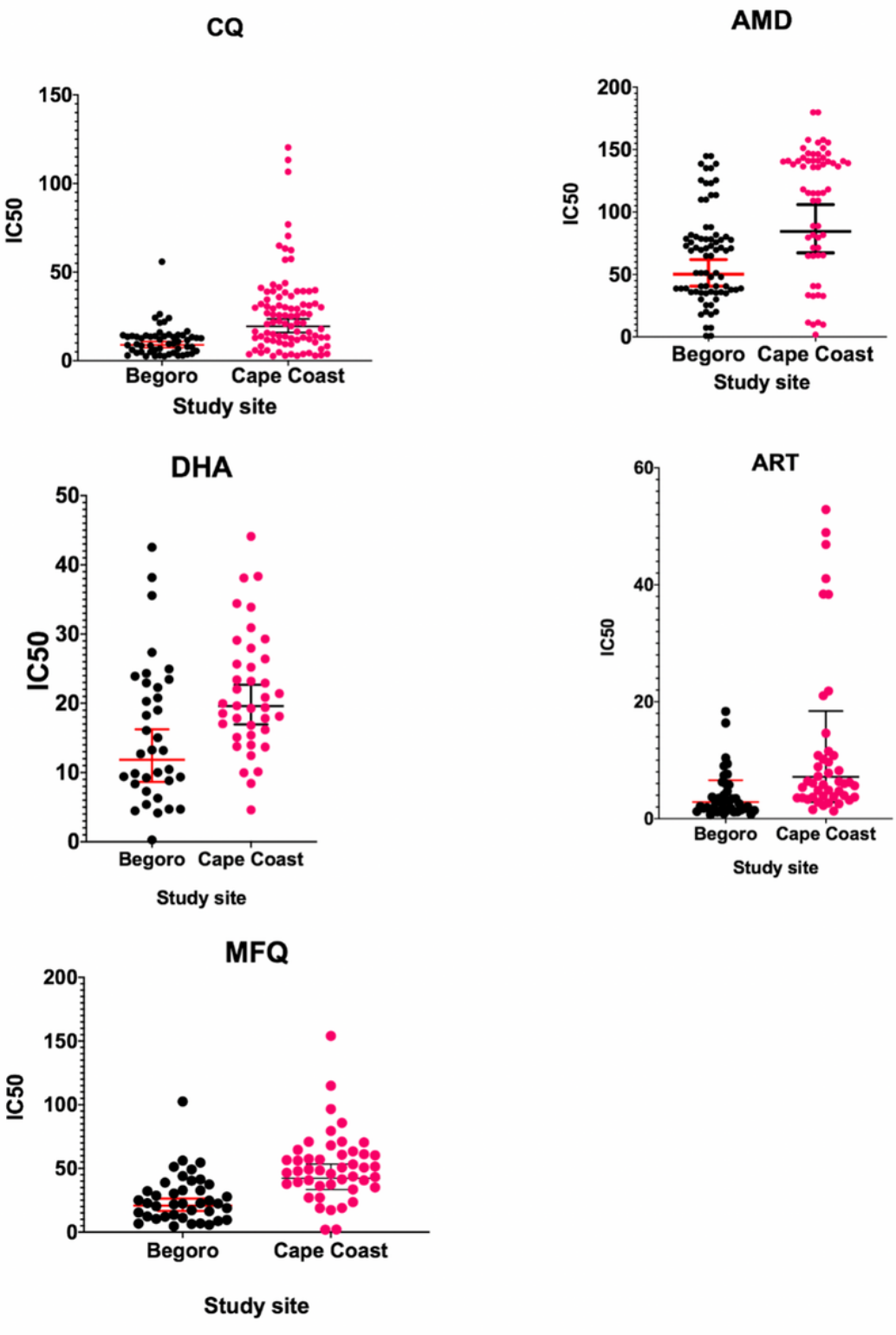

Figure 3

Distribution of IC50 values of Chloroquine (CQ), Amodiaquine (AMD), Dihydro-Artemisinin (DHA), Artesunate (ART) and Mefloquine (MFQ against P. falciparum parasites collected from Cape Coast and Begoro in 2017. The black and pick dots represent individual IC50 values for parasites collected from Cape Coast and Begoro respectively and the horizontal lines represent the geometric mean of IC50 with their respective 95% CI.

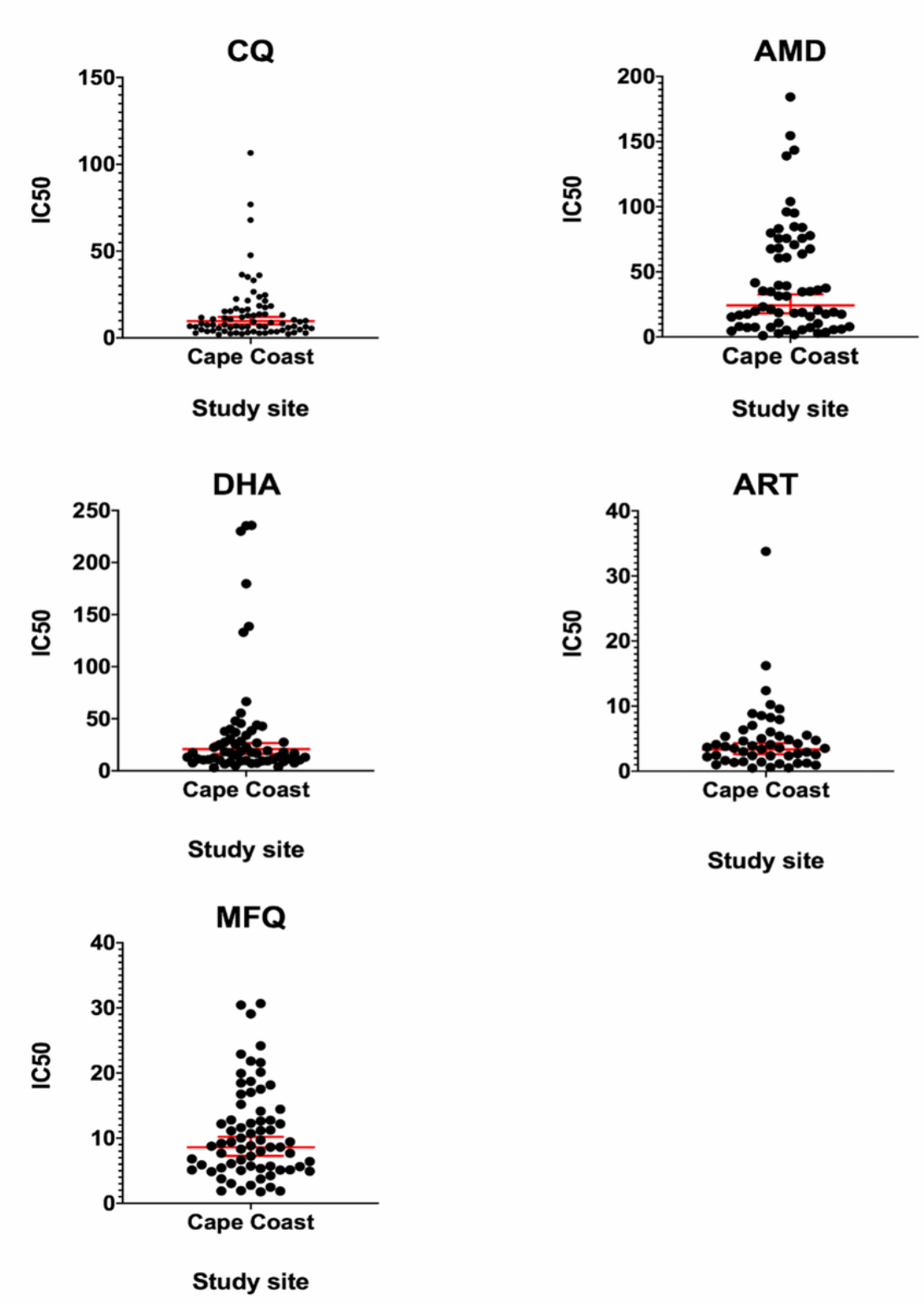

Figure 4

Trends of geometric mean of IC50 of all the five drugs against the parasites collected from a) Cape Coast from 2015 to 2017 and b) Begoro from 2016 to 2017.