Kinetics of *Plasmodium falciparum* Gametocyte Sex Ratios: Application to the Evaluation of the Potential of Antimalarial Drugs to Influence Malaria Transmission

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Abstract: A non-compartment pharmacokinetic model was used to evaluate the potential of antimalarial drugs to influence malaria transmission using the ratio of sex specific gametocyte half-lives male:female- the gametocyte maleness index (GMI), and the ratio of the sex specific area inscribed by the plot of gametocyte sex density *versus* time curves AUCmg:AUCfg- the gametocyte maleness load index (GMLI). Data on gametocyte sexes collected in children with symptomatic *Plasmodium falciparum* malaria who were treated with various antimalarial drugs in an endemic area were examined using the two indices. Both GMI and GMLI were positively correlated ($r = 0.71$, $P < 0.0001$) and with 95% limits of agreement of -9.7 to 13.8 using Altman-Bland plot. Based on the assumption that, a male-biased sex ratio, if gametocytaemia is low, may increase mosquito infectivity, both GMI and GMLI, consistently gave index ratios > 1 for 4-aminoquinolines and antifolates suggesting potential for increasing the chance of mosquito infectivity. By contrast, artesunate and artemisinin-based combination therapies (ACTs), artemether-lumefantrine, and artesunate-amodiaquine, and a non-ACT, amodiaquine plus sulfalene-pyrimethamine, had ratios < 1 suggesting potential for reducing the chance of mosquito infectivity. The advantages and drawbacks of using these indices as tools in assessing the influence of antimalarials on transmission potentials in endemic areas of malaria are discussed.

Keywords: *P. falciparum*, gametocytes, sex ratio, transmission, antimalarials, children, Nigeria.

INTRODUCTION

Gametocytes, the sexual forms of *Plasmodium spp*., are essential for the transmission of the parasite from the vertebrate host to humans by mosquitoes obtaining a human blood meal and for the infectivity of the vertebrate host obtaining the blood meal. Mosquito infectivity after a blood meal can be influenced by a number of factors including gametocyte density [1], their sex ratios [2, 3] defined as the proportion of peripheral gametocytes that are male [4], and the type of antimalarials used [5-7].

If the transmission success of *Plasmodium spp*. is threatened by host and other factors, the parasite may respond by investing in more gametocyte production, for example, *P. chabaudi* or by increasing the sex ratio of its gametocytes, for example, *P. vinckei petteri* [8]. Recent studies have shown that in human malarias, antimalarials may increase gametocyte production and sex ratios, for example, *in vitro* and *in vivo* by *P. falciparum* [9-12].

Current methods for evaluation of mosquito infectivity after a blood meal have employed detection and estimates of sporozoites load in laboratory bred, human blood fed mosquitoes [2, 6, 7, 13]. Although these methods are adequate and can be used to assess the influence of antimalarials on mosquito infectivity [7] they are laborious, time consuming and require considerable expertise and may not be optimal in epidemiological settings. In addition, mosquito infectivity studies have not employed pharmacokinetic principles. If gametocyte sex ratio is crucial to mosquito infectivity and, if antimalarials significantly alter gametocyte density and sex ratio, then there is an urgent need to explore alternative, simple, and complimentary methods for the evaluation of the potential of antimalarial drugs to influence malaria transmission in epidemiological settings. Further more, transmission is a dynamic process and residents of endemic areas constantly self medicate for imagined or proven malaria infections.

In order to address these issues in an area of intense transmission, we have developed new indices based on pharmacokinetic principles, for assessing the influence of antimalarial drugs on gametocyte sexes and used them to evaluate the potential of these drugs to enhance or reduce malaria transmission. The indices are based on the assumption that male-biased gametocyte sex ratio is crucial to mosquito infectivity [2], particularly when gametocytaemia is low (<10/μl blood) [14]. Our aims were to: use a non-compartment pharmacokinetic model to evaluate the disposition of gametocyte sexes and, to develop new indices to evaluate the influence of antimalarials on transmissibility based on the changes in gametocyte sex ratio. We applied the indices to the data on gametocyte sexes collected from a series of antimalarial studies in children treated with various antimalarial drugs in an endemic area of malaria in southwest Nigeria between 1999 and 2006 and compared the agreement between the two indices.

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Table 1. Treatment Regimens of the Children Enrolled in the Study

Drugs*	Regimens†
CQ	30 mg/kg of chloroquine base over 3 days, that is, 10 mg/kg daily
AQ	30 mg/kg of amodiaquine base over 3 days, that is, 10 mg/kg daily
PS	Pyrimethamine-sulphadoxine given as 25 mg/kg of the sulphadoxine component at presentation
COT	Cotrimoxazole given as 25 mg/kg of the sulphamethoxazole component twice daily for 5 days
AS	Artesunate given as 28 mg/kg over 7 days, that is, 4 mg/kg daily
PSP	Pyrimethamine-sulphadoxine given as in PS above plus probenecid at 20-25mg/kg in two divided doses daily for 3 days
AL	Artemether (20mg) plus lumefantrine (120mg) given thus: 5-14kg received 1 tab., 15-24kg received 2 tab., 25-34kg received 3 tab., > 34kg received4 tab. at presentation 8hr later and at 24, 36, 48 and 60hrs after first dose
ASAQ	Artesunate given as in AS above plus amodiaquine given as in AQ above
ASP	Amodiaquine given as in AQ above plus sulfalene-pyrimethamine given as 25 mg/kg of the sulfalene component

* 162, 648, 184, 53, 120, 78, 90, 183 and 91 children were enrolled in CQ, AQ, PS, COT, AS, PSP, AL, ASAQ and ASP groups, respectively.
† All drugs were administered orally.
Abbreviations: CQ, chloroquine; AQ, amodiaquine; PS, pyrimethamine-sulphadoxine; COT, cotrimoxazole; AS, artesunate; PSP, pyrimethamine-sulphadoxine plus probenecid; AL, artemether plus lumefantrine; ASAQ, artesunate plus amodiaquine; ASP, amodiaquine plus sulfalene-pyrimethamine

PATIENTS AND METHODS

Patients

Patients were recruited from 1999-2006 at the malaria clinic of the University College Hospital in Ibadan, southwest Nigeria, an endemic area of malaria [15] into various antimalarial efficacy studies and were enrolled if the following criteria were met: an age 0.5-14 years, fever or history of fever in the 24-48 h preceding presentation, pure *Plasmodium falciparum* parasitaemia $\geq$ 2000/µl blood, absence of concomitant illness, negative urine tests for 4-aminoquinoline (Dill- Glazko) and sulfonamides (lignin), and written informed consent of a parent or guardian. Patients with severe malaria [16] or serious underlying diseases (renal, cardiac or hepatic) or severe malnutrition were excluded from the study. The studies received approval from the local ethics committee.

Drug Management, and Quantification and Determination of Gametocyte Sex

Drug treatment was according to standard schedules [10, 17, 18] see Table **1** for detail). At enrolment (day 0) and at follow-up on days 1-7, 14, 21, and 28 (up to 2003) and on 1-3, 7, 14, 21, 28, 35 and 42 (after 2003), patients underwent full physical examination and thin and thick blood films examination for quantification of asexual and sexual parasitaemia. Quantification of asexual and sexual parasites in thick films was done against 500 and 1000 leukocytes, respectively assuming a leukocyte count of 6000/µl blood. All gametocytes were sexed if gametocytaemia $\geq$ 10/µl blood and according to the following criteria [19]: males (microgametocytes) are smaller than females (macrogametocytes), the nucleus is larger in males than females, the ends of the cells are rounder in males and angular in females, with Giemsa the cytoplasm stains purple in males and deep blue in females, and the granules of malaria pigment are centrally located in females and more widely scattered in males. The sex ratio was defined as the proportion of gametocytes in peripheral blood that were male [4]. Blood obtained from a finger prick into heparinized capillary tubes was used to estimate the haematocrit.

Kinetics of Sex Specific Gametocytaemia

Gametocyte kinetic parameters were estimated from gametocyte and sex-specific densities (gametocyte and gametocyte sex concentrations or gametocytaemias) by a non compartment model using the computer programme *Turbo Ken* (Clinical Pharmacology Group, University of Southampton, UK, through the courtesy of Professor A.G. Renwick) as previously described [12]. Briefly, the following parameters were calculated from the curve of sex specific gametocytaemia by using the real times of sampling from each patient: areas under the curves of gametocytaemia *versus* time until the last detectable gametocyte concentration (C_{tgm}), (AUC_{gmlast}), were calculated using the trapezoidal method. Area under the sex specific gametocytaemia-time from zero to infinity ($AUC_{gm0-\infty}$) was calculated by adding to AUC_{gmlast} the extrapolated AUC_{gm} calculated as C_{tgm}/k_{el}, the elimination rate constant derived from the semilogarithic plot of sex specific gametocytaemia *versus* time. In this context, visual inspection of the final part of the gametocytaemia-time curve was used to identify the elimination phase. Terminal sex specific elimination half life, $t_{1/2ß}$, was calculated as $0.693/k_{el}$. The final sex specific gametocytaemia at the apparent time of clearance was taken to be 0.001sexual forms/µl blood (a level assumed to be below microscopical detection). Sex specific areas under the curve and half-lives were determined only in patients who had gametocytaemia at enrolment and for at least three times during the first 7-14 days after enrolment.

Definition of New Indices, GMI and GMLI

Gametocyte maleness index (GMI) was defined as the ratio of sex specific half lives male : female. Gametocyte maleness load index (GMLI) was defined as the ratio of sex specific areas under the curve male : female, that is AUCmg (male gametocyte) : AUC fg (female gametocyte). These ratios were determined for each drug treatment group. Patients participating in GMI and GMLI evaluation were 50

randomly selected children who had gametocytaemia >10/µl blood at presentation

DATA ANALYSIS

Data were analyzed using version 6 of the *Epi-Info* software [20], the statistical programme *SPSS for Windows* version 10.01 [21] and *MedCalc* version 9.4.0.0 [22]. Variables considered in the analysis were related to the densities of *P. falciparum* gametocytes and trophozoites. Proportions were compared by calculating χ^2 with Yates' correction or by Fisher exact or by Mantel Haenszel tests. Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney U-tests and the Kruskal-Wallis tests (or by Wilcoxon ranked sum test). Association between GMI and GMLI was derived from each patient by correlation analysis and agreement between the two methods of measuring gametocyte maleness was further examined by Altman-Bland analysis [23]. All tests of significances were two-tailed. P-values of $\leq$ 0.05 were taken to indicate significant differences. Data were (double)-entered serially using the patients' codes and were only analyzed at the end of the study.

RESULTS

Patient Characteristics at Enrolment

The clinical and parasitological characteristics of the children enrolled in the efficacy studies and those from whom the new indices were derived are shown in Table **2**. The gametocyte sex ratio in the cohort of 162 children who carried gametocytes at enrolment was 0.18 $\pm$ 0.02 (SEM, standard error of mean) and in the subset of 52 children in whom GMI and GMLI were evaluated was 0.31 $\pm$ 0.03 (SEM).

Table 2. Clinical and Parasitological Characteristics of Children Enrolled and those who Met the Criteria for the Gametocyte Maleness Load Index (GMLI) and Gametocyte Maleness Index (GMI)

Characteristic	Total Children Enrolled (1999-2006)	Children who Participated in GMLI and GMI Evaluation††
Number	1609	52
Age (years)	6.08 $\pm$ 3.09	4.89 $\pm$ 2.76
Gender: female/male	791 / 725	26 / 26
Weight (kg)	17.22 $\pm$ 6.58	15.08 $\pm$ 5.28
Duration of illness (days)	2.96 $\pm$ 1.29	3.06 $\pm$ 1.43
Temperature (°C)	38.20 $\pm$ 1.18	37.78 $\pm$ 1.03
Packed cell volume (%)	30.54 $\pm$ 3.88	27.54 $\pm$ 5.42
Parasitaemia* (/µl blood)	33583 (1052 - 6194285)	22919 (1352 - 1376551)
Gametocytaemia* (/µl blood)	18 (6 - 498)	39 (6 - 498)
Gametocyte sex ratio†	0.18 $\pm$ 0.02 (n = 162)	0.31 $\pm$ 0.04

All values are mean $\pm$ standard deviation
* Values are geometric mean (range)
† Values are mean $\pm$ standard error of mean
††Patients were randomly selected

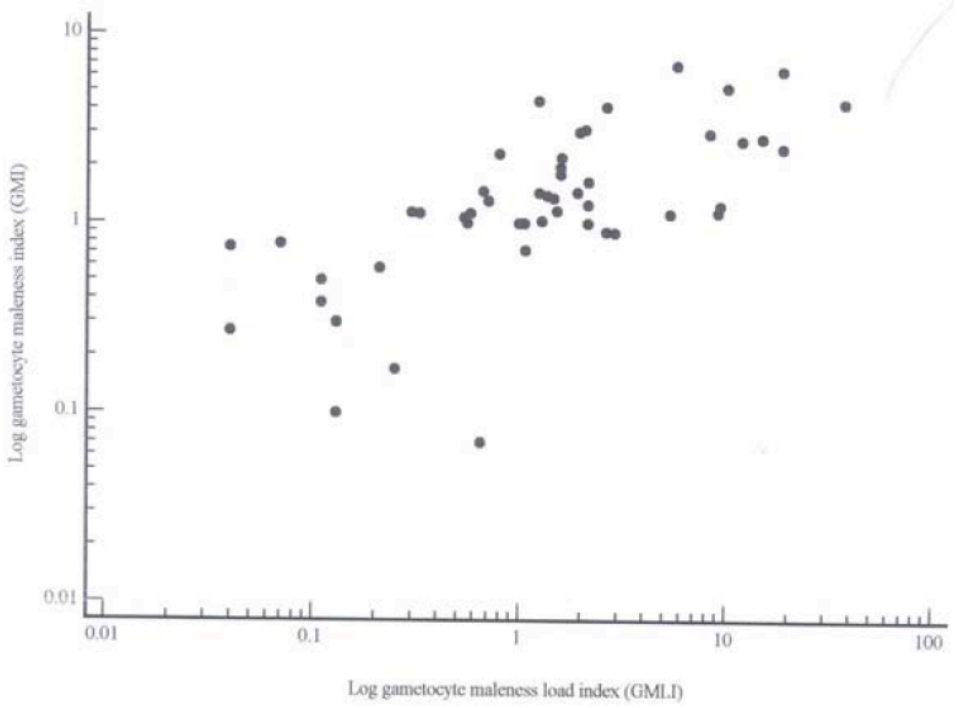

Fig. (1). Scatter plot of log gametocyte maleness index (GMI) and log gametocyte maleness load index (GMLI) in children with falciparum malaria treated with antimalarial drugs (n = 52, r = 0.71, P < 0.0001).

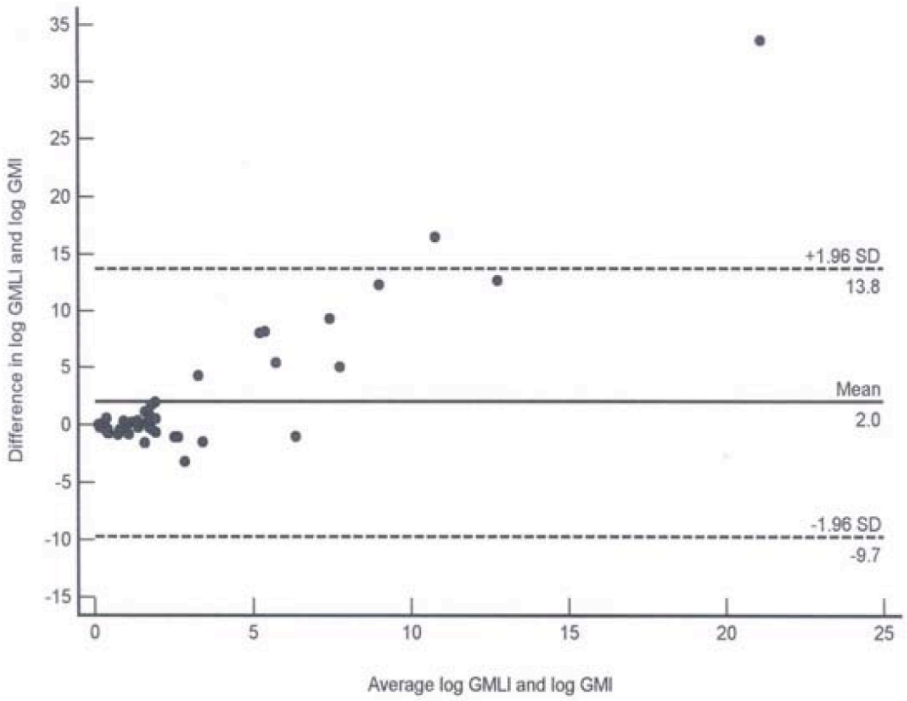

Fig. (2). Altman-Bland plot of log gametocyte maleness index (GMI) and log gametocyte maleness load index (GMLI) in children with falciparum malaria treated with antimalarial drugs. The mean (± standard deviation, SD) value of the difference is shown.

Association between GMI and GMLI in Individual Patient

Fig. (**1**) is a scatter plot of GMI and GMLI values in patients in whom both data were available. There was a significant correlation between the two methods ($r = 0.71$, $P < 0.0001$, $n = 52$).

Agreement between GMI and GMLI in Individual Patients

The Altman-Bland plot of the difference between log GMI and log GMLI against the average of both values are shown in Fig. (**2**). The limit of agreement for the two methods was –9.7 to 13.8. The 95% confidence interval (95%CI for the upper limit of agreement was 10.9 to 16.6 and for the lower limit of agreement was –12.5 to –6.8.

Drug Related GMI and GMLI

The GMI and GMLI values for each treatment groups are shown in Tables **3** and **4**, respectively. Because sex specific AUCs (not half-lives) were not normally distributed, geometric mean values are indicated for GMI and GMLI on Tables **3** and **4**. In general, values of GMI and GMLI were similar for each drug treatment group, and are significantly correlated ($r = 0.9$, $P = 0.001$) and close to unity.

DISCUSSION

Current methods for the measurement of malarial transmission have not taken into full account the potential of antimalarial drugs to enhance or reduce malaria transmission in endemic communities and have not used models based on pharmacokinetic principles. We have evaluated a kinetic

Table 3. Sex Specific Half-Lives Following Treatment with Antimalarial Drugs and Drug Specific Gametocyte Maleness Index (GMI)

Drug Treatment	Half-life (days) Microgametocyte	Macrogametocyte	GMI
CQ	0.80 (13) 0.30 – 1.03	0.33 (13) 0.13 – 1.03	2.27
AQ	0.74 (17) 0.16 – 3.77	0.56 (17) 0.15 – 1.03	1.32
PS	0.96 (5) 0.55 – 2.56	0.50 (5) 0.13 – 1.12	1.92
COT	0.74 (2) 0.73 – 0.75	0.30 (2) 0.14 – 0.66	2.47
AS	0.21 (5) 0.08 – 0.82	0.67 (5) 0.31 – 1.45	0.31
PSP	0.40 (3) 0.08 – 0.93	0.21 (3) 0.08 – 0.34	1.90
AL	0.27 (2) 0.08 – 0.88	0.59 (2) 0.30 – 1.17	0.46
ASAQ	0.74 (3) 0.52 – 1.03	1.67 (3) 1.03 – 3.50	0.44
ASP	0.32 (2) 0.15 – 0.68	0.86 (2) 0.85 – 0.86	0.37

All values are geometric mean (number) range
GMI, gametocyte maleness index defined as the ratio of sex specific half lives male: female
CQ, chloroquine; AQ, amodiaquine; PS, pyrimethamine-sulphadoxine; COT, cotrimoxazole; AS, artesunate; PSP, pyrimethamine-sulphadoxine plus probenecid; AL, artemether plus lumefantrine; ASAQ, artesunate plus amodiaquine; ASP, amodiaquine plus sulfalene-pyrimethamine

Table 4. Sex Specific AUCs Following Treatment with Anti-malarial Drugs and Drug Specific Gametocyte Maleness Index (GMLI)

Drug Treatment	AUC (μl^{-1}.day) Microgametocyte	Macrogametocyte	GMLI
CQ	158 (13) 12.45 – 1926.82	59 (13) 6.69 – 259.89	2.68
AQ	236 (17) 6.69 – 3566.06	187 (17) 9.35 – 2879.80	1.26
PS	1456 (5) 214.73 – 4689.95	324 (5) 73.90 – 2257.23	4.49
COT	231 (2) 164.33 – 326.23	24 (2) 17.80 – 31.84	9.62
AS	27 (5) 6.69 – 60.75	98 (5) 10.28 – 274.26	0.28
PSP	158 (3) 9.35 – 881.04	26 (3) 9.35 – 46.35	6.08
AL	7 (2) 0.68 – 66.75	168 (2) 15.61 – 1801.02	0.04
ASAQ	94 (3) 51.29 – 190.22	661 (3) 400.89 – 1485.30	0.14
ASP	35 (2) 18.35 – 66.74	256 (2) 72.66 – 902.56	0.14

All values are geometric mean (number) range
AUC, area under the curve of gametocytaemia *versus* time; GMLI, gametocyte maleness load index, defined as the ratio of sex specific AUC male: female; CQ, chloroquine; AQ, amodiaquine; PS, pyrimethamine-sulphadoxine; COT, cotrimoxazole; AS, artesunate; PSP, pyrimethamine-sulphadoxine plus probenecid; AL, artemether plus lumefantrine; ASAQ, artesunate plus amodiaquine; ASP, amodiaquine plus sulfalene-pyrimethamine

approach to malaria transmission based on the assumption that gametocyte sex ratio is crucial to mosquito infectivity and derived two new indices for quantification of the potential of antimalarial drugs to enhance or reduce malaria transmission in endemic communities. Our methods have distinct advantages: they employed the levels of gametocyte found in peripheral blood over a relatively long period of time and allow a quantitative approach; the sampling periods far exceeded the estimated gametocyte sequestration time of 4 days *in vivo* in patients not previously exposed to malaria [24] and the average estimated half life of gametocytaemia in children resident in endemic areas [10-12]. Additionally, the methods allow assessment of the effects of each drug on gametocyte sexes and their application is independent of any definition of a male-biased sex ratio. It is noteworthy that the software for pharmacokinetic analyses of half life and areas under the curves of gametocyte density *versus* time are readily available. The main drawback of the methods is that they require careful follow up of patients over a period of 1-2 weeks, a situation that may be difficult in patients who self medicate. In addition only subjects in whom there was gametocytaemia at enrolment could be used to calculate these indices. A population pharmacokinetic approach may be applied to resolve the problems posed by the last.

The most important findings of the present study are the wide range of the 95% CI for agreement between the pharmacokinetic indices at the individual level and the considerable agreement for the estimation of the effects of antimalarial drugs at the group level. The clinical significance of the discrepancy between the two methods at the individual and group levels is unclear. The narrow limits of agreement between the two methods at the group level would suggest that either method is sufficiently reliable for independent use. However, it is our opinion that GMI has less variation and may be a relatively simpler method to use.

Both the half life and the area inscribed by the plot of gametocyte density against time are kinetically related. However, $AUC_{gmsexes}$ is a quantitative measure of load carried over a period of time. In this context, it is a measure of investment, and when related to gametocytes, relative investment in the sexes. Thus it has been suggested [18] that in areas of intense transmission and depending on the availability of both gametocyte sexes, it is likely that the proportion of mosquitoes which become infected following a blood meal after treatment may be related to the AUC_{gm} providing both gametocyte sexes are available and viable. This proportion may also be expected to be high if more male gametocytes are present in the blood meals. Thus high GMLI after treatment may provide a quantitative estimate of the influence of drugs on the potentials for transmissibility.

Analyses of drug-related effects using the values of derived from both methods were similar. In this context, the data suggest that *P. falciparum*, when exposed to anti-malarials, which threatens its survival invest in gametocyte sex ratio following treatment with 4-aminoquinolines and antifolates. In addition these drugs also increases gameto-cytaemia and carriage [11, 25, 26] suggesting investment in gametocytes. By contrast treatment with artesunate and ACTs was not associated with investment in gametocytes or sex ratio. These findings would suggest antimalarials have differing effects on the responses of *P. falciparum* on its survival strategies.

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CONFLICT OF INTEREST

None Declared.

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