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ORIGINAL ARTICLE

Effectiveness of Artemether Lumefantrine and Dihydroartemisinin Piperaquine in Clearance of Gametocytes in Uncomplicated *Plasmodium falciparum* Malaria in Tiwi Kenya

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ABSTRACT

Background: Over 80 countries worldwide have now implemented WHO recommendations to use Artemisinin-Based Combination Therapy as a first-line treatment for ***Plasmodium falciparum*** malaria. The sexual stage of ***P. falciparum*** is responsible for the transmission of malarial parasites to infectious mosquitoes. Studies on gametocytes are generally based on microscopic detection, which is not sensitive, and there is a need for more sensitive molecular techniques that can detect and quantify gametocytes at densities as low as 0.02 to 0.1 gametocytes per micro-litre. The objective of this study was to determine the clearance rates of gametocytes after AL and DHA&P in uncomplicated ***P. falciparum*** and to compare the effectiveness of microscopy and reverse transcriptase-polymerase chain reaction in gametocyte detection.

Methods: In a randomised controlled clinical trial of samples collected, gametocyte densities were quantified by microscopy by counting against 500 leukocytes in the thick smear converted to numbers of parasites per micro-litre by assuming a standard count of 800 leukocytes per micro-litre of blood after staining with 10% Giemsa stain and by reverse transcriptase-polymerase chain reaction using primers specific to the pfs25 gene.

Results: There was no significant difference between the drug's gametocyte clearance ($p<.082$). The drugs cleared gametocytes in infected patients by day 28 as detected by microscopy. There was a significant difference in the detection of gametocytes by RT-PCR and microscopy ($p<.001$).

Conclusion: This study showed that Artemether-Lumefantrine and Dihydroartemisinin piperaquine have gametocytocidal effects on ***P. falciparum*** and the study on the clearance of gametocytes using both artemether-lumefantrine and Dihydroartemisinin piperaquine may be carried out using a larger sample size for policy implementation. The reverse transcriptase-polymerase chain reaction is more effective than microscopy in detecting low levels of gametocytes and the pfs25 gene can be used in the detection of gametocytes in the field to monitor the clearance of gametocytes.

INTRODUCTION

Malaria is a global life-threatening disease in humans caused by 5 species of *Plasmodium* parasites, *Plasmodium vivax, P. ovale, P. malariae, P. falciparum,* and *P.* knowlesi.[1] Globally, there were an estimated 241 million malaria cases in 2020. This represents about 14 million more cases in 2020 compared to 2019 and 69,000 more deaths.[2] Approximately two-thirds (47,000) of these deaths were linked to disruptions in the provision of malaria prevention, diagnosis, and treatment during the pandemic.

The mortality rate due to malaria in children aged under 5 years had reduced from 87% in 2000 to 77%. In 2020, malaria deaths increased by 12% compared with 2019, to an estimated 627 000; an estimated 47 000 (68%) of the additional 69 000 deaths were due to service disruptions during the COVID-19 pandemic.[2] In Kenya, malaria remains a leading cause of morbidity and mortality, especially in young children and pregnant women. It accounts for 30% of outpatient attendances and 19% of admissions to health facilities in endemic areas.[2] The prevalence of Malaria in Kenya was at 1.1% by the year 2020 according to the 2021 world malaria report 2021.[2] The Kenya malaria indicator survey conducted by the Ministry of Health (MOH) in 2020 reported Malaria prevalence at 8%[3]. In Kenya, Artemether Lumefantrine and Dihydroartemisinin-piperaquine are used as the first and second-line drugs respectively for the treatment and management of malaria cases.

The malaria control program in Kenya has been scaling up vector control interventions, timely diagnosis, and effective treatment of malaria using Artemisinin-Based Combination Therapy (ACT), and Intermittent Preventive Treatment for Pregnant Women (IPTP). Only the asexual parasite load causes the symptomatic disease; antimalarial drugs are primarily active against this stage, although some are-

also active against developing or mature gametocytes (gametocytocidal), and some may also disrupt the development of the ookinete in the mosquito gut.[3]

Gametocytocidal activity is conventionally regarded as advantageous because it may have a public health benefit in decreasing transmission. It is hypothesized that killing the transmission stages will reduce the rate at which resistance spreads.[4] The National Malaria Strategy adopted 4 interventions which include providing the right drugs at the right time, protecting pregnant women, promoting distribution and use of insecticide-treated nets, and pre-empting epidemics.[4] The transmission of malaria depends on the presence of mature sexual stage parasites or gametocytes in human peripheral blood.

The emergence and spread of *P. falciparum* resistance to antimalarial drugs are one of the greatest challenges facing global efforts to control malaria. To prevent drug resistance, there is clear evidence that combining more than one drug can improve its efficacy without increasing toxicity.[5] The development of highly effective artemisinin derivatives offers hope for the treatment of malaria using Artemisinin-Based Combination Therapy. The Artemisinin-Based Combination Therapy prevents individual resistance to individual drugs by relying on the principle of combining2 drugs with different mechanisms of action.[6] The fast-acting artemisinin derivative rapidly clears the main parasite load within a few hours to its therapeutic levels and thus reducing subsequent gametocyte carriage.[7] The partner drug, which is generally longer lasting, clears the rest of the parasites. In this study, we used 2 different artemisinin combination drugs: Artemisinin-Lumefantrine (AL) and Dihydroartemisinin-piperaquine (DHA&P) for the detection of clearance of gametocytes using microscopy and quantitative PCR.

MATERIALS AND METHODS

Study Site

The study was conducted in Tiwi in Kwale County of Coastal Kenya. This region has continuous malaria transmission with children and pregnant women being the most vulnerable group.[8] Malaria spread in the region is largely restricted to the long rainy season (May-June) and unpredictable short rainy seasons (October-November). This study was conducted from May to June during the rainy season when parasite density rises due to the rains that provide good breeding sites for mosquito vectors. The average annual rainfall of the region is 508 mm (10 years average). The entomological inoculation rate in the region has never been estimated.

Study Design

The study was a randomised clinical trial with a minimum of 116 participants. A randomisation list of how the 2 study drugs were allocated was computer-generated by an off-site investigator. Sequentially, numbered, and sealed envelopes containing the treatment group assignments were prepared from the randomisation list.

Study Population and Sampling Procedures

The study subjects were recruited from malaria patients who visited Tiwi Health Centre. The recruitment was based on the inclusion and exclusion guidelines for the assessment and monitoring of antimalarial drug efficacy for the treatment of uncomplicated *P. falciparum* malaria.[9] The study nurses assigned treatment numbers sequentially and allocated treatment by opening the envelope corresponding to the treatment number. Only the study nurse was aware of the treatment assignments. All other study personnel, including the study physicians and laboratory personnel involved in assessing outcomes, were blinded to the treatment assignments. The patients were not informed of their treatment regimen.

Ethical Consideration

The study protocol (SSC No. 1955) was approved by the Scientific Steering Committee and the Ethical Review Committee of the Kenya Medical Research Institute. Written informed consent was obtained from the patients, and for participants below 18 years, the parents or guardians consented on their behalf. Only those who consented were recruited for the study. The slide and filter paper samples were given codes to conceal the identities of the patients. Patients were explained in detail the entire study procedure including the need to voluntarily participate, the anticipated benefits and/or risks, and the duration of involvement in the study. The risk of participation in this project was minimal. The possible risks of drawing blood included infection, bruising, and bleeding.

Inclusion Criteria

Those who consented and met the inclusion criteria were recruited for the study. The study subjects were children aged 6 months to10 years with uncomplicated *P. falciparum* malaria with no other *Plasmodium* species present using light microscopy, having an initial parasite density of 500- 100,000 asexual parasites/µl, having a measured axillary temperature of ≥37.5°C ≤ 39.5°C, no history of antimalarial drug intake during the previous month, providing informed consent (by parent or guardian, where appropriate) and willingness to return for follow-up.

Exclusion Criteria

Children who reported treatment with antimalarial chemotherapy 2 weeks before recruitment, those who experienced persistent and severe malaria, and evidence of a chronic disease or an acute infection other than malarial parasites, were residing outside Tiwi were excluded.

Patient Recruitment and Follow-up

In total, 200 cases suspected of uncomplicated malaria were screened for eligibility to participate in the study during an 8-week recruitment period, in May and June 2011. A total of 84 children were excluded because they did not meet the inclusion criteria. A total of 116 patients fulfilled the inclusion criteria and were recruited of which 58 were randomly administered DHA&P and, 58 AL.

Sample Collection

Blood samples were collected via a finger prick and spotted on a slide from the study subjects and followed up on days 3, 7, 14, and 28 for microscopic examination. Subsequently, from the finger prick, a drop of blood was collected on filter paper on the days scheduled for follow-up for molecular studies. The filter papers were air-dried

and stored in a zip-lock plastic bag with desiccators for molecular analysis. Study participants received the same treatment regimen for all subsequent episodes of malaria. The reference drugs were administered according to weight-based guidelines for fractions of tablets as follows: AL was given twice daily for 3 days. One tablet of DHA&P was given once per day for 3 days. Patients were given a glass of milk or requested to be breastfed after each dose of study medication. The first daily dose of study drugs was directly observed for 30 min at the study clinic, and the dose was re-administered if vomiting occurred.

Laboratory Procedures

Finger prick blood samples (50 µl) for RT-PCR analysis were collected on Cytiva Whatman 903™ protein saver card 1, manufacturer Cytiva US filter papers, and air-dried at room temperature. Nucleic acid extraction was performed as described by Bousema et al.[10] Total RNA was isolated using a High Pure RNA isolation kit.[11] (Roche, Lewes UK) Gametocyte-infected blood obtained from an *in vitro* culture of *P. falciparum* 3D7 clone was used as the positive control. Filter papers spotted with 50µl of *Plasmodium*-negative whole blood were used as negative controls for all steps of analysis. Briefly, the columns were centrifuged at 12,000 rpm for 2 minutes at room temperature. Some of RNA was used to make cDNA while the rest was stored at -80^{0}C.

Amplification of the pfs 25 Gene

The 21 µl mix for each reaction tube was made consisting of; 3µl of 10µm dNTPs, 8µl of 1× RT buffer 1µl of anti-sense primer, 1 µl of reverse transcriptase enzyme, and 8 µl of RNA and incubated at 37^{0}c for 45 minutes in a thermocycler[12], 7 µl cDNA was added to a 23 µl master mixture containing 100 µM of each dNTP, buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$), 0.3 µl of Pfs25F (5′- atcgatATGAATAAACTTTACAGTTTGTTTCT-3′), 0.3 µl of Pfs25R, (5′-T7-CATTTACCGTTACCACAAGTTA-3′), 14.36 µl of ddH_2O and 0.24 µl of enzyme Taq polymerase. The PCR cycling conditions were for 25 cycles. Initial denaturation at 94°C for 2 minutes, denaturation at 94°C for 30 seconds, annealing at 50°C for 35 seconds, and extension at 68°C for 2.5 minutes. 2 µl of the product of the first PCR was used as a template for a nested PCR using a set of internal primers sense 25-1, (5′-TAATGCGAAAGTTACCGTGG-3′) anti-sense 25-2(5′CCATCAACAGCTTTACA GG-3′).

Agarose Gel Electrophoresis

The amplicons of pfs 25 gene products were resolved by electrophoresis on a 2.0% agarose gel, stained with ethidium bromide, run for 30 minutes at 80 volts and the product was estimated by comparison to gel pilot mid-range ladder (100) molecular weight marker (Qiagen®) North American, the US in Germantown, Maryland, run in the adjacent lane. The presence of a 500bp band representing amplification of the Pfs25 gene was determined using ultraviolet illumination and a digital camera.

Statistical Analysis

Data was entered in an excel spreadsheet and analysed by Stata 16. The prevalence of gametocytes was calculated by the number of positive patients with gametocytes divided by the total number of patients positive with malaria parasites. The student's t-test was used to analyse the clearance rates of gametocytes by both drugs using gametocyte density while the Chi-square was used to analyse the prevalence data of gametocytes detected by both techniques from days 0, 3, 7, 14, and 28.

RESULTS

Table 1 shows the mean gametocyte density (g/µl) detected by microscopy in the different study groups in patients treated with AL and DHA&P at different time intervals. In general, both drugs cleared all gametocytes by day 28. The number of gametocytes decreased to zero by day 28 but the difference was not significant ($p<.082$).

Prevalence Rates

The presence of gametocytes in clinical samples was assessed by microscopy and RT-PCR. Table 2 shows the prevalence at different times in 2 groups detected by microscopy. On day 0, gametocyte prevalence by microscopy was 12(24.0%) in the group treated with DHA&P and 4(8.3%) in the group treated with AL (p=.036). On day 3, the gametocytes prevalence was 11(22.0%) in the group treated with DHA&P and 3(6.2%) in the group treated with AL (p=.026). On day 7, the gametocyte prevalence was 4(8.0%) in the DHA&P and 2(4.2%) with AL (p=.678) while on day 14, the gametocyte prevalence was 0(0.0%) in the DHA&P and 1(2.1%) with AL(p=.490). On day 28 gametocytes were not detected by microscopy in both groups treated with DHA&P and AL.

Table 3 Shows gametocyte prevalence detected by RT-PCR. The prevalence was 24(48.0%) in the group treated with DHA&P and 31(64.6%) in the group treated with AL (p=.098). On day 3, the prevalence was 19(39.6%) and 30(60.0%) in the AL and DHA&P groups respectively (p=.043). On day 7, the prevalence was more than twice in the group treated with DHA&P compared with 12(25.0%) in the AL group (p=.002) while on day 14 the prevalence was 10(20.8%) in the group treated with AL and 30.0% in the group treated with DHA&P (p=.298). On day 28, the prevalence was 6.3% in the group treated with AL and 10% in the group treated with DHA&P (p=.715).

Evaluation of the Effectiveness of Microscopy and RT-PCR in the Detection Of gametocytes

Table 4 shows the total gametocyte prevalence detected by microscopy and RT-PCR. Overall, RT-PCR was able to detect significantly more cases of malaria in day 0, 55(56.1%) vs. 16(16.3%); $p<.001$, day 3 49(50.0%) vs. 14(14.3%); $p<.001$, day 7 40(40.8%) vs.6(6.1%); p<.001, day 14 25(25.5%) vs. 1(1.0%); p<.001, and in day 28 8(8.2%) vs. 0.0%; $p=.003$). On average, RT-PCR detected 10 times more gametocytes compared to microscopy and the difference between the methods in the detection of gametocytes was significant.

DISCUSSION

During the period of malaria infection cases, gametocytes are responsible for the transmission of malaria from infectious female anopheles' mosquitoes to human beings.[13] In this study, the effectiveness of AL and DHA&P

TABLE 1: Mean Number of Gametocytes (g/µl) Detected by Microscopy in the Different Study Groups

Day of examination	AL		DHA&P		
	n	Mean+SE	N	Mean+SE	p-value
Day 0	4	112+21	12	92+10	0.363
Day 3	4	64+29	12	63+10	0.956
Day 7	4	24+15	12	17+8	0.696
Day 14	4	8+8	12	0+0	0.082
Day 28	4	0+0	12	0+0	N/A

TABLE 2: Gametocyte Prevalence (%) by Microscopy on the Different Follow-Up Days

Day of examination	MICROSCOPY				
	AL (n=48)		DHA&P (n=50)		
	n	%	N	%	*p- value*
Day 0	4	8.3	12	24.0	.036
Day 3	3	6.2	11	22.0	.026
Day 7	2	4.2	4	8.0	.678
Day 14	1	2.1	0	0.0	.490
Day 28	0	0.0	0	0.0	N/A

TABLE 3: Gametocyte's Prevalence (%) by RT-PCR in the Different Follow-Up Days

Day of examination	RT-PCR				
	AL (n=48)		DHA&P (n=50)		
	n	%	N	%	*p -value*
Day 0	31	64.6	24	48.0	.098
Day 3	19	39.6	30	60.0	.043
Day 7	12	25.0	28	56.0	.002
Day 14	10	20.8	15	30.0	.298
Day 28	3	6.3	5	10.0	.715

TABLE 4: Total Gametocyte Prevalence (%) Detected by Microscopy and RT-PCR

Day of examination	MICRO (n=98)		RT-PCR (n=98)		
	n	%	N	%	p-value
Day 0	16	16.3	55	56.1	<0.001
Day 3	14	14.3	49	50.0	<0.001
Day 7	6	6.1	40	40.8	<0.001
Day 14	1	1.0	25	25.5	<0.001
Day 28	0	0.0	8	8.2	<0.003

in the clearance of gametocytes of *P. falciparum*, the effectiveness of RT-PCR and microscopy in the detection of gametocytes, and the detection of the pfs25 gene of the gametocytes were evaluated.

Results showed no difference in the clearance of gametocytes by both drugs. This is because according to statistical analysis performed, there was no significant difference even though in the table results section, few gametocytes were reported in the 2 drugs that were used for the treatment of patients. The drugs cleared gametocytes in positive patients by day 28. These findings are similar to findings reported by Petra *et al.*[14] who found that clearance of gametocytes in patients treated with DHA&P had no significant difference with patients treated with AL. Both drugs rapidly clear parasitemia and fever, and demonstrate a significant gametocidal effect, even in areas of widespread parasite resistance to other antimalarial.[15]

In this study, the 2 drugs showed similarities concerning their effectiveness and clearance of gametocytes compared with other studies conducted elsewhere[16,17,18]. However, most of these studies had a follow-up of 42 days which is different from the 28 days follow-up in this study. This could have been the reason for the difference in the results observed. Despite the effectiveness of AL, there were substantial limitations to this regimen, including; twice-daily dosing and the need for administration with fatty food. However, many other studies have analysed the efficacy of AL and DHA&P in the clearance of gametocytes, and all reported very good results.[19, 20, 21, 22]

This study showed less effectiveness of DHA&P on gametocyte clearance in comparison with AL when a more sensitive RT-PCR test was used for gametocyte detection. This could limit the effectiveness of DHA&P in areas with low malaria transmission. However, this finding should be further investigated in larger studies in different study sites with different transmission intensities. Using RT-PCR, gametocytes were present in low numbers throughout the 28 days of follow-up in both study groups. Previous studies have shown that both drugs can reduce malaria transmission in the community.[19,23] However, the 90% gametocytaemia clearance cited in these studies were observed > 20 days post-treatment. In our study, both groups of patients had < 5% gametocytaemia on day 14 post-treatment. Artemisinin derivatives kill young gametocytes.[24] This may explain the persistence of gametocytes after 3- and 7-day courses of treatment in uncomplicated malaria.

Prolonged gametocytaemia has been proposed as an early sign of the emergence of antimalarial drug resistance.[25] This might be a concern given the poor gametocytocidal effects of DHA&P. However, gametocyte density remained low, and the gametocyte clearance was fast. During the 28 days of follow-up, few patients had gametocytes in this study, which reflects the good gametocytocidal properties of the Artemisinin-based combination therapy. However, artemisinin-based combination therapy has, in general, a negative effect on gametocyte development and survival and thus influences malaria transmission, at least in low transmission areas.[26, 27, 28] In this study, there was a significant difference in the detection of gametocytes with RT-PCR and microscopy. On day 28 of the follow-up period, RT-PCR detected up to 10 times more gametocytes confirming that RT-PCR was more effective than microscopy. RT-PCR detection techniques have demonstrated that gametocytes can be seriously underestimated using microscopy. In this study, RT-PCR gave estimates of gametocyte prevalence 10-fold higher than microscopy. This compares well with other studies which recorded ten times higher gametocytes than estimated by microscopy.[29] The detection of gametocytes by microscopy is insufficiently sensitive to assess potential infectivity. Gametocyte densities below the microscopic threshold for gametocyte detection (~ 5 gametocytes/µl) frequently result in mosquito infection.[30] This study showed that with sensitive detection RT-PCR, a difference in gametocyte clearance can be observed but these results should be confirmed in larger studies and in other study areas with different malaria transmission intensities.

The RT-PCR detected the *P*fs25 gene using primers specific for this gene with the presence of approximately 500 base pair bands representing the amplification of this gene. The RT-PCR detected the presence of gametocytes in positive malaria patients, and this showed it can be applied to guide case management in the control of malaria transmission. RT-PCR is reliable in determining the prevalence data of gametocyte carriage in the population, needed to know the infectious reservoir and battle the ongoing transmission of malaria.[31]

The RT-PCR was able to detect gametocytes below the threshold of microscopic detection and is highly specific for its gametocyte targets and also in the presence of a vast excess of asexual forms[32], as shown in this study where it detected gametocytes below the threshold of microscopic detection. The RT-PCR had a detection limit of 20 to 100 gametocytes/mL of blood, and the high-throughput format allows its use in the assessment of gametocyte carriers in the population, and it is critical in understanding malaria transmission dynamics in epidemiological studies.[33,34] A previous study with RT-PCR showed a very high prevalence of gametocytes in symptomatic children in Kenya.[35] The RT-PCR detection of gametocytes enables the treatment of carriers to clear parasitaemia and reduce the source of infection available to mosquitoes that emerge at the start of the rainy season. This could contribute to malaria control strategy if high coverage with effective therapy is achieved.

CONCLUSIONS

This study showed that AL and DHA&P have gametocytocidal effects on *P. falciparum.* The RT-PCR is more effective than microscopy in the detection of low levels of gametocytes. The pfs25 gene can be used in the detection of gametocytes in the field to monitor the clearance of gametocytes. The findings of this study can be used as policy guidelines in rolling out mass artemisinin-based combination therapy to reduce the gametocyte prevalence in asymptomatic and symptomatic patients to prevent and control malaria cases. It is critical in understanding malaria transmission dynamics in epidemiological studies.

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