Evidence of an increased incidence of day 3 parasitaemia in Suriname: an indicator of the emerging resistance of *Plasmodium falciparum* to artemether

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The emerging resistance to artemisinin derivatives that has been reported in South-East Asia led us to assess the efficacy of artemether-lumefantrine as the first line therapy for uncomplicated Plasmodium falciparum *infections in Suriname. This drug assessment was performed according to the recommendations of the World Health Organization in 2011. The decreasing number of malaria cases in Suriname, which are currently limited to migrating populations and gold miners, precludes any conclusions on artemether efficacy because adequate numbers of patients with 28-day follow-up data are difficult to obtain. Therefore, a comparison of day 3 parasitaemia in a 2011 study and in a 2005/2006 study was used to detect the emergence of resistance to artemether. The prevalence of day 3 parasitaemia was assessed in a study in 2011 and was compared to that in a study in 2005/2006. The same protocol was used in both studies and artemether-lumefantrine was the study drug. Of 48 evaluable patients in 2011, 15 (31%) still had parasitaemia on day 3 compared to one (2%) out of 45 evaluable patients in 2005/2006. Overall, 11 evaluable patients in the 2011 study who were followed up until day 28 had negative slides and similar findings were obtained in all 38 evaluable patients in the 2005/2006 study. The significantly increased incidence of parasite persistence on day 3 may be an indication of emerging resistance to artemether.*

Key words: artemether - artemisinin resistance - *Plasmodium falciparum* - day 3 parasitaemia - Suriname

The emergence of *Plasmodium falciparum* parasites that are resistant to artemisinin derivatives was first reported along the Cambodia-Thailand border. This resistance threatens the sustained malaria control that has been achieved in recent years (Noedl et al. 2008, Dondorp et al. 2009, 2011, WHO 2011). Monotherapy, suboptimal dosing and non-adherence are factors that contribute to this emerging resistance (Dondorp et al. 2009, 2011, Bethell et al. 2011). Recent studies have revealed a similar trend of resistance to artemisinin derivatives in Thailand (Phyo et al. 2012). Furthermore, a study from the Kenyan coast suggested a reduced sensitivity to artemisinin derivatives (Borrmann et al. 2011). In South America, *P. falciparum* field isolates from French Guyana were reported to have reduced in vitro sensitivity to artemether (Jambou et al. 2005). However, high susceptibility to dihydroartemisinin and lumefantrine compounds has been observed in Columbia and both Suriname and Brazil have reported excellent efficacy rates with artemisinin derivatives (Aponte et al. 2011, Gama et al. 2011, Vreden et al. 2011). Because artemisinin-based combination therapies (ACT) are the standard treatment for *P. falciparum* malaria, more efforts are needed to intensify malaria control and to implement additional treatment strategies (WHO 2011).

There are conflicting reports on the association between mutations in the *Pf*ATP6 and *Pf*MDR1 genes and a reduced sensitivity to artemisinin derivatives (Pickard et al. 2003, Cheeseman et al. 2012, Pillai et al. 2012). Additionally, no standardised in vitro tests are available to assess artemisinin resistance (Dondorp et al. 2011, WHO 2012). Determining parasite clearance rates, particularly the half-life slope, is a reliable method to assess resistance to artemisinin derivatives, but require frequent blood sampling (Flegg et al. 2011). Ideally, patients should be treated in an experimental hospital setting where they receive artemisinin monotherapy, e.g., artesunate, for seven days, followed by the administration of an additional drug, e.g., mefloquine (Dondorp et al. 2011). However, this approach may not be feasible for studying emerging resistance in mobile populations. Because artemisinin is highly active against young circulating ring-stage parasites before cytoadherence occurs, the presence of parasitaemia on day 3 post-infection can be used as an indicator of the reduced sensitivity of *P. falciparum* parasites to treatment with artemisinin derivatives. In addition, evaluating parasitaemia on day 3 requires less frequent blood sampling (Stepniewska et al. 2010, White 2011). Day 3 parasitaemia may be a poor predictor of patient outcomes on day 28 because the supplemental drug may still clear the infection. However, determining the presence of day 3 parasitaemia is the most useful method for assessing artemisinin resistance in mobile populations.

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Artemisinin resistance is confirmed when treatment failure occurs after treatment with oral artemisinin-based monotherapy. Treatment failure is defined as persisting parasitaemia on day 7 post-infection or recrudescence within 28 days after a positive result for parasitaemia on day 3. Artemisinin resistance is suspected when an increased parasite clearance time is observed. Resistance is defined as the presence of 10% of cases or more with detectable parasites on day 3 after treatment with ACT (WHO 2011).

Malaria control activities have increased in Suriname since 2004, which include the introduction of artemether-lumefantrine as the first line treatment of uncomplicated *P. falciparum* infection. These activities led to a decline in the incidence of malaria from more than 16,000 cases in 2001 to 806 cases in 2011 and a decline in cases due to *P. falciparum* (WHO 2010b). The first assessment of artemether-lumefantrine as a first line regimen in 2005/2006 indicated an efficacy of more than 95% (Vreden et al. 2011). Determining the presence of resistance to artemether-lumefantrine is a key concern because of global development and the influx of mobile gold miners in Suriname who have a history of poor adherence and widespread use of illegally acquired antimalarial drugs. An insufficient number of *P. falciparum* cases that could be followed for 28 days was expected; therefore, determining day 3 parasitaemia was welcomed as an alternative for the initial assessment of decreased sensitivity to artemisinin derivatives. We designed a study protocol in 2011 and compared the results to those from an efficacy study that was performed in 2005/2006. In addition, the study assessed day 3 parasitaemia to detect trends of reduced sensitivity to artemether in Suriname.

PATIENTS, MATERIALS AND METHODS

Study site - Suriname is situated in the northeast region of South America and is bordered by French Guiana to the east, Brazil to the south and Guyana to the west (ABS 2005). Suriname has a population of approximately 500,000 individuals and 80% of its territory is covered by tropical rainforest. Approximately 62,000 people live in small settlements that are scattered throughout the Amazon rainforest. In recent years, the population in the rainforest has increased by approximately 15,000 people, due to small-scale gold mining (Heemskerk 2009). The transmission of malaria through *Anopheles darlingi* occurs only in the interior of the country. The transmission of malaria occurs mainly in mobile gold mining communities, especially along the border with French Guiana (Veiga 1997, Heemskerk 2009, Hiwat et al. 2012). This study was conducted in Paramaribo, the capital of Suriname, at the Tourtonne Laboratory from April 2011-November 2011. This malaria diagnosis and treatment facility is located in an area of the city where most of the mobile gold miners reside during their short stays in the capital. More than 80% of all malaria cases in Suriname are diagnosed at this laboratory. Approval was granted by the Ministry of Health to conduct this study after the protocol was evaluated by the ethics board.

Study population - The study population consisted of males and females two years of age or older with microscopically confirmed *P. falciparum*. To detect the presence of parasites on day 3 in 10% of cases or more with a 5% level of significance and 80% power, at least 60 patients needed to be enrolled. This calculation compensated for a 20% loss to follow-up (WHO 2003, 2009). Eligible patients had a sole *P. falciparum* infection with initial parasite densities that ranged from 200-100,000 asexual parasites/mm^3 as determined by microscopy using the same protocol as used in the efficacy study in 2005/2006 (Vreden et al. 2011). According to this protocol, the entire smear was first screened at a low magnification (10X, 40X objective lens) to detect suitable fields with an even distribution of white blood cells (10-20 white blood cells/field). The smears were examined using a 100X oil immersion lens. At least 500 high-power fields were examined before a thick smear was declared negative. *P. falciparum* parasites were counted per 200 leukocytes (or 500 leukocytes when less than 11 parasites were counted per 200 leukocytes). The parasite density per microlitre of blood was estimated by assuming a leukocyte count of 8 x 10^9/L. Eligible patients had a fever or a history of fever within 24 h in the absence of another cause or chronic medical condition. Patients were eligible to participate in the study when they were available to be directly observed during treatment and could attend follow-up visits for at least three days. Written informed consent was obtained from the patient or legal guardian. Pregnant women and patients with signs of severe malaria or a history of hypersensitivity reactions to any of the drugs being tested were excluded from the study.

Initial assessment and follow-up - Clinical, demographic, haematological and parasitological assessments were performed on day 0. Urine pregnancy tests were performed for the women. Standard clinical and parasitological monitoring occurred on days 2 and 3 and, when possible, on days 7, 14, 21 and 28. Haematological monitoring occurred on day 2 and, when available, on day 28. Additional follow-up was conducted between visits and after day 28 when the patients felt unwell or still had parasitaemia on day 3. Patients were withdrawn from the study when they had signs of treatment failure, developed severe or complicated malaria, self-administered additional antimalarial drugs, had signs of persistent vomiting, required a blood transfusion, developed allergic reactions to artemether-lumefantrine or were lost to follow-up.

Drug therapy - Coartem® (Novartis AG, Basel, Switzerland) with a fixed ratio of 20 mg/120 mg was started on day 0 and was administered orally twice daily in dosages that were recommended by the WHO for three days (WHO 2010c). The first dose was consistently administered within 2 h after the day 0 slide was drawn. The administration of the drug and a 30-min observation occurred under the supervision of clinical staff. In cases of vomiting, 100% of the original dose was re-dosed during this time. Primaquine (0.75 mg/kg) was administered on day 2. Additional antipyretics (in the case of an axillary temperature > 39ºC) and

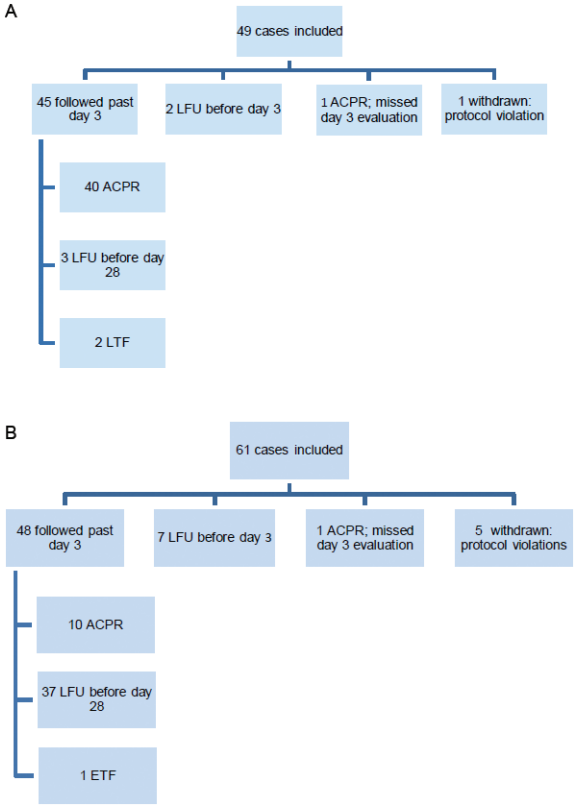
antiemetics were given when vomiting occurred during the first dose of treatment.

Outcome measures - A 0 parasite count was interpreted as parasite densities that fell below the detection level of 50 parasites/mm^3. Parasite densities for days 0, 2 and 3 and the percentage of patients with parasitaemia (PPP) on days 2 and 3 [PPP48 (48 h) and PPP72 (72 h) respectively] were calculated. The 28-day follow-up results were reported when available; however, these findings were not a clinical endpoint in this study. Similar data from the 2005/2006 study were included to allow for a comparison and a statistical analysis.

Statistical analysis - Student's *t* test, the Mann-Whitney *U* test, Fisher's exact test and chi-squared tests were used for the statistical analysis of the data from 2005/2006 and 2011. The analysis was performed using SPSS v.17.0. p-values < 0.05 were considered statistically significant.

RESULTS

During the enrolment period from April 2011-November 2011, 74 patients were diagnosed with *P. falciparum* malaria. Of these patients, 61 met the inclusion criteria (Figure). Overall, 48 of these patients could be followed until day 3 and 10 patients could be followed until day 28. One patient who could not be evaluated on day 3 was followed up through day 28. Figure shows the number of patients who were included in the 2005/2006 study of artemether-lumefantrine in which a higher rate of patients who were followed up until day 28 was observed (82% vs. 16%; p < 0.001) (Table I).



A: follow-up diagram for all consenting patients from day 0 until day 28 in 2005/2006 from the artemether-lumefantrine arm (A) and from day 0 until day 28 in 2011 (B). ACPR: adequate clinical and parasitological response; ETF: early treatment failure; LFU: lost to follow-up; LTF: late treatment failure.

Demographic characteristics - No significant differences were observed in age, the male to female ratio or weight between the efficacy study in 2005/2006 and the study that was undertaken in 2011 (Table I). The mean ages of the study populations were 30 ± 15 years and 31 ± nine years for the 2005/2006 and 2011 studies, respectively. In 2011, the study population consisted of adults only. However, the 2005/2006 study included children. The mean body weights were 62.0 ± 19.5 kg and 63.4 ± 11.2 kg in the 2005/2006 and 2011 studies, respectively. In the 2011 study, most of the participants reported that they were working in gold mines on the French Guiana-Suriname border. In contrast, more patients in the 2005/2006 study were from villages in the interior of Suriname.

Clinical outcomes - No adverse events were reported during treatment. All 11 patients who were followed up until day 28 in the 2011 study exhibited an adequate clinical and parasitological response (ACPR) compared with 40 of 42 patients in the 2005/2006 study. In the 2005/2006 study, two cases of late treatment failure were observed. These cases were positive for parasites on days 22 and 28 after being negative on day 2. One case in the 2011 study had a higher parasite density on day 2 compared with day 0, which indicated early treatment failure (ETF) (Figure).

Parasite clearance - The geometric mean of parasitaemia on day 0 was significantly higher in the 2005/2006 study than in the 2011 study (9,588.98 and 2,757.88 asexual parasites/mm^3, respectively, p < 0.005) (Table II). The PPP48 in the 2011 study was 75% and was significantly higher than the PPP48 in the 2005/2006 study, which was 18% [p < 0.001, odds ratio (OR) 13.5, 95% confidence interval (CI) 4.932-36.949]. The PPP72 in the 2011 study was 31% and was significantly higher than the PPP72 in the 2005/2006 study, which was 2% (p < 0.001; OR 20, 95% CI 2.514-159.130) (Table II). In the 2011 study, no significant differences were observed in the initial parasitaemia between patients with day 3 parasitaemia and patients without day 3 parasitaemia (p = 0.747).

DISCUSSION

A small number of patients could be followed until day 28, which prohibited conclusions on the efficacy of artemether-lumefantrine for the treatment of *P. falciparum* in Suriname. The majority of the study population in 2011 came from small-scale gold mining communities, which explains the lower follow-up rate because gold miners tend to remain in the city for only a few days. Therefore, this study emphasises the use of alternative methods for assessing artemisinin sensitivity.

We observed lower rates of initial parasitaemia in the 2011 study compared with the 2005/2006 study, which is in contrast to reports from Nigeria where the geometric

mean parasite density increased significantly after the introduction of artemisinin derivatives (Gbotosho et al. 2011). The increased awareness of *P. falciparum* infection and the improved access to diagnosis and treatment in Suriname may explain this difference.

The log-reduction in parasitaemia over time, which is the definition of the parasite clearance rate, is considered a first order process (Flegg et al. 2011). Because different parasite densities can be found at the initiation of antimalarial therapy due to different stages of parasite development, an initial plateau has been observed in approximately 30% of cases when plotting the log-parasitaemia over time. Using an average duration of 6 h, this plateau is referred to as the lag phase and is excluded when determining parasite clearance rates (White 2011). Additionally, pre-treatment parasite densities influence parasite clearance times. Determining the half-life slope, which is independent of initial parasitaemia, can therefore be used to assess parasite clearance. An overestimation of the half-life slope occurs when 12 h measurements are obtained (Flegg et al. 2011). The limited number of samples (< 3 positive parasite counts) in the 2005/2006 and 2011 studies and the broad intervals between sampling in both studies limit the estimation of the parasite clearance rates and the half-life slope.

The parasitaemia on day 2 was higher in the 2011 study. Parasitaemia ratios at 48 h can be used for assessing drug efficacy regardless of how often blood smears are collected (White 1997). As shown in Table II, significantly more patients in the 2011 study remained positive for parasites on days 2 and 3 compared with the 2005/2006 study despite receiving identical treatment. The presence of parasites on day 3 was observed in 31% of the cases in the 2011 efficacy study compared with 2% of the cases (1 patient) in the 2005/2006 study ($p < 0.001$). Therefore, a statistically significant increase in the incidence of day 3 parasitaemia occurred in the 2011 study. The number of evaluable patients in this study was lower than the number that was indicated in our sample size calculation; therefore, the percentage of parasitaemia on day 3 suggested that the level of significance and power were maintained.

Greater proportions of patients with artemisinin-sensitive *P. falciparum* parasites on day 3 have higher pre-treatment parasitaemia (Stepniewska et al. 2010). Such data are useful in the interpretation of day 3 parasitaemia when assessing artemisinin resistance. The proportion of patients who were positive for parasites on day 3 in the 2011 study was above the normal distribution with a 99% CI (Stepniewska et al. 2010, White 2011). Therefore, these data strongly suggest a reduced sensitivity to artemether.

TABLE II

Parasitological assessment days 0, 2 and 3

	2005/2006	2011	
Day 0 parasitaemia[a]			
Geometric mean	9,588.98	2,757.88	$p < 0.001$[b]
Range	512-82,057	215-89,880	-
Median	12,541.00	2,445.00	-
25th percentile	4,616.00	682.75	-
50th percentile	12,541.00	2,445.00	-
75th percentile	24,879.50	8,524.00	-
Interquartile range	20,263.5	7,841.25	-
Patients with parasitaemia on days 2 and 3 (%)			
PPP48	18 (8/44)[c]	75 (36/48)	$p < 0.001$[d]
PPP72	2 (1/45)	31 (15/48)	$p < 0.001$[d]

a: asexual parasites/mm^3; *b*: Mann-Whitney *U* test; *c*: data from one patient in 2005/2006 on day 2 was missing and was therefore excluded when determining the percentage of patients with parasitaemia at 48 h (PPP48); *d*: Fisher's exact test; PPP72: PPP on day 3 at 72 h.

TABLE I

Characteristics study population 2005/2006 and 2011

	2005/2006	2011	
Males vs. females (%)[a]	71 vs. 29	65 vs. 35	p = 0.501
Age (years)[b]	30 ± 15	31 ± 9	p = 0.600
Age groups (years) (%)[a]			
< 5	2	0	p = 0.018
5-15	13	0	
> 15	84	100	
Weight (kg)[b]	62.0 ± 19.5	63.4 ± 11.2	p = 0.688
Follow-up rate until day 3 (%)[a]	92	79	p = 0.07
Follow-up rate until day 28 (%)[a]	82	16	p < 0.001

a: chi-square analysis; *b*: Student's *t* test.

No definite correlation has been detected between ACPR and day 3 parasitaemia. All of the patients in the 2005/2006 and 2011 studies who could be followed until day 28 exhibited an ACPR.

Day 3 parasitaemia is indicative of artemisinin resistance; however, this measuring point is primarily indicated to exclude artemisinin resistance (Stepniewska et al. 2010, White 2011). The presence of 10% of cases or more with parasitaemia on day 3 after a three-day regimen with ACT requires further evaluation (Stepniewska et al. 2010, WHO 2010a). The majority of participants in the 2011 study reportedly worked in gold mines along the French Guiana-Suriname borders. The working conditions and health behaviours do not differ on either side of the border. The use of illegally acquired antimalarial drugs in gold mines is prevalent in Suriname. However, a pharmaceutical analysis revealed that dihydroartemisinin in illegally acquired ACTs in Suriname, mostly Artecom (dihydroartemisinin/piperaquine), passed quality tests (Evans et al. 2012). When malaria is suspected, gold miners tend to take half of the antimalarial treatment and save the remaining tablets for future bouts of fever due to limited financial resources; therefore, the development of resistance can be ascribed to poor adherence rather than the use of ineffective illegally acquired drugs. The study population in 2005/2006 included people from rural villages in the interior of the country; therefore, the malaria parasites in the gold miners may not differ from those in individuals from the villages because the patients in both studies resided in the same geographical area along the French Guiana-Suriname border. The mobility of these small gold mining communities can further increase the spread of malaria (Heemskerk 2009). Despite the small study population in the 2011 efficacy study, the results from 2011 compared with those from the 2005/2006 study suggest an emerging resistance to artemether. These results do not contradict the finding that isolates that were obtained before or after adopting artemether-lumefantrine as the first line regimen in Suriname do not exhibit any mutations in the *Pf*ATP6 gene (Adhin et al. 2012). However, our results suggest that single nucleotide polymorphisms in the *Pf*ATP6 gene are not directly associated with artemether resistance.

We observed an increase in the rate of day 3 parasitaemia from 2-31% over a five-year time span. Our study supports the use of day 3 parasitaemia as a tool for screening artemisinin resistance, particularly in the absence of functional molecular markers and standardised in vitro test systems. The WHO advises that an incidence of day 3 parasitaemia that exceeds 10% should prompt governments to take measures to prevent the development of treatment failure (WHO 2010a). A study has recently been initiated in Suriname to evaluate parasite clearance rates during artemisinin monotherapy and this study should confirm whether our findings can be ascribed to decreased artemisinin sensitivity.

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