Am. J. Trop. Med. Hyg., 97(2), 2017, pp. 526–532
doi:10.4269/ajtmh.16-0780

Sustained High Cure Rate of Artemether–Lumefantrine against Uncomplicated *Plasmodium falciparum* Malaria after 8 Years of Its Wide-Scale Use in Bagamoyo District, Tanzania

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Abstract. We assessed the temporal trend of artemether–lumefantrine (AL) cure rate after 8 years of its wide-scale use for treatment of uncomplicated *Plasmodium falciparum* malaria from 2006 to 2014 in Bagamoyo district, Tanzania. Trend analysis was performed for four studies conducted in 2006, 2007–2008, 2012–2013, and 2014. Patients with acute uncomplicated *P. falciparum* malaria were enrolled, treated with standard AL regimen and followed-up for 3 (2006), 28 (2014), 42 (2012–2013), or 56 (2007–2008) days for clinical and laboratory evaluation. Primary outcome was day 28 polymerase chain reaction (PCR)-adjusted cure rate across years from 2007 to 2014. Parasite clearance was slower for the 2006 and 2007–2008 cohorts with less than 50% of patients cleared of parasitemia on day 1, but was rapid for the 2012–2013 and 2014 cohorts. Day 28 PCR-adjusted cure rate was 168/170 (98.8%) (95% confidence interval [CI], 97.2–100), 122/127 (96.1%) (95% CI, 92.6–99.5), and 206/207 (99.5%) (95% CI, 98.6–100) in 2007–2008, 2012–2013, and 2014, respectively. There was no significant change in the trend of cure rate between 2007 and 2014 (χ^2_{trend} test = 0.06, $P = 0.90$). Pretreatment *P. falciparum* multidrug-resistant gene 1 (*Pfmdr1*) N86 prevalence increased significantly across years from 13/48 (27.1%) in 2006 to 183/213 (85.9%) in 2014 ($P < 0.001$), and *P. falciparum* chloroquine resistance transporter gene (*Pfcrt*) K76 prevalence increased significantly from 24/47 (51.1%) in 2006 to 198/205 (96.6%) in 2014 ($P < 0.001$). The AL cure rate remained high after 8 years of its wide-scale use in Bagamoyo district for the treatment of uncomplicated *P. falciparum* malaria despite an increase in prevalence of pretreatment *Pfmdr1* N86 and *Pfcrt* K76 between 2006 and 2014.

INTRODUCTION

Artemisinin–based combination therapy (ACT) is the recommended first-line treatment of uncomplicated *Plasmodium falciparum* malaria globally.[1] Tanzania adopted artemether–lumefantrine (AL) as first-line treatment of uncomplicated malaria in November 2006.[2] The drug has shown to have high cure rate both in Tanzania and other parts of Africa with most of the countries indicating a failure rate of < 5%.[3] Follow-up studies conducted in Bagamoyo district, and other parts of Tanzania between 2007 and 2013 have also reported an AL failure rate of ≤ 4%.[4–6] However, following frequent exposure to ACT, *P. falciparum* parasite biology and probably its susceptibility to the drug have been changing.[7,8]

Plasmodium falciparum resistance against ACTs has emerged in parts of southeast Asia (SEA),[9,10] and it has been associated with *Pfkelch*13 mutations.[11,12] In Africa, no ACT resistance has been reported; however, there are reports of reduced in vitro sensitivity of *P. falciparum* parasites against lumefantrine, the long-acting partner drug of AL.[13–15] The SEA ACT-resistance-associated polymorphisms have, however, not been reported in Africa.[16,17] Conversely, selection of single nucleotide polymorphisms in *P. falciparum* multidrug-resistant gene 1 (*Pfmdr1*) N86 and chloroquine resistance transporter gene (*Pfcrt*) K76 has been reported after treatment with AL both in vivo and in vitro, and is thought to be associated with the reduced parasite sensitivity against lumefantrine.[13–15,18–20] A temporal trend analysis of data collected in Bagamoyo district between 2004 and 2011 revealed an increase in the proportion of *Pfmdr1* N86 and *Pfcrt* K76 in the parasite population following the adoption of AL policy, and a corresponding decrease in the proportion of *Pfmdr1* 86Y and *Pfcrt* 76T, which were associated with chloroquine resistance.[7] However, it is not well understood whether the increase in *Pfmdr1* N86 and *Pf*crt K76 prevalence has affected the AL cure rate in this area. Temporal trend analysis might help to monitor the AL cure rate in relation to *Pfmdr1* N86Y and *Pf*crt K76T changes over time in the study area.

Therefore, the aim of this study was to assess the temporal trend of the AL cure rate and its association with baseline *Pfmdr1* N86Y and *Pf*crt K76T before and after 8 years of wide-scale use of the ACT as first-line treatment of uncomplicated *P. falciparum* malaria in Bagamoyo district, Tanzania.

MATERIALS AND METHODS

Study area, design, and population. The trials were conducted at Fukayosi and Yombo primary health facilities, Bagamoyo district, Tanzania, between 2006 and 2014. Fukayosi and Yombo health facilities serves around 10,000 and 7,000 people, respectively. Both facilities have ability to carry out routine malaria microscopy and rapid diagnostic test.

Bagamoyo district is a high endemic area with malaria transmission occurring throughout the year with peaks related to the long rain season from May to July and short

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rain season from November to December. *Plasmodium falciparum* and *Anopheles gambiae* sensu stricto are the major malaria parasite species and vector, respectively.[21,22] AL is used as the first-line treatment of uncomplicated malaria in Tanzania since November 2006. Long-lasting insecticide-treated mosquito nets is the major vector control method.[23]

The first trial was an AL pharmacokinetics and pharmacodynamics study conducted in 2006,[24] the second trial was a supervised treatment arm of the two arms AL efficacy and effectiveness clinical trial carried out in 2007–2008,[4] the third trial was an AL efficacy trial conducted in 2012–2013 (unpublished data), and the fourth trial was a two arms AL and AL plus a single low-dose primaquine (PQ) efficacy and safety clinical trial carried out in 2014.[25] The PQ arm was included since there was no statistically significant difference in the cure rate between AL and AL + PQ arm.[26] The first study was conducted 6 months before implementation of AL treatment policy, whereas the second, third, and fourth studies were conducted 1, 6, and 8 years after the implementation of AL in Bagamoyo district, respectively. The subjects inclusion and exclusion criteria are described elsewhere.[4,24,25]

Patients with microscopically confirmed *P. falciparum* infection were enrolled, admitted during the first 3 days, treated and then followed up for 3 (2006), 28 (2014), 42 (2012–2013), or 56 (2007–2008) days for clinical and laboratory evaluation. However, for the current study, the treatment outcomes were assessed by day 28.

Based on treatment response, the patients were classified as having therapeutic failure: early treatment failure, late clinical failure (LCF), late parasitological failure (LPF), or polymerase chain reaction (PCR)-adjusted adequate clinical and parasitological response.[27]

Patients treatment and procedure. Enrolled patients were treated with a standard 3 days course of AL (Coartem®, Novartis Pharma, Basel, Switzerland) according to Tanzanian national treatment guidelines for uncomplicated *P. falciparum* malaria with the second dose administered exactly 8 hours after the first dose and the remaining doses administered in the morning and evening,[2] but a slight modification was done for the 2006 cohort and a subset of 45 patients from the 2012–2013 cohort, whereby doses were given at 0, 8, 24, 36, 48, and 60 hours. For the 2014 cohort, a 0.25 mg/kg single-dose PQ was administered together with the first AL dose among patients in the AL + PQ arm.[25] All doses were directly observed. Patients were observed for 30 minutes after each drug dose and treatment was readministered in case of vomiting within this period.

Patients were followed up on days 0, 1, 2, and 3 for the 2006 study; 0, 1, 2, 3, 7, 14, 21, 28, or any day of recurrent illness for the 2014 study; 0, 1, 2, 3, 7, 14, 21, 28, and 42 for the 2012–2013 study; and 0, 1, 2, 3, 7, 14, 21, 28, 35, 42, 49, and 56 for the 2007–2008 study. However, a slight modification of the follow-up schedule was done for the 2006 cohort and the subset of 45 patients from the 2012–2013 cohort for which assessments were done during the early phase of treatment at 0, 4, 8, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, and 72 hours to assess parasite clearance time. Patients who missed a scheduled follow-up visit between day 7 and 56, and did not show-up despite efforts to trace them were considered lost to follow-up and consequently withdrawn.

Laboratory assessment involved collection of finger-prick blood samples for haemoglobin concentration, thick blood smears for microscopy determined asexual and sexual parasitemia, and filter papers (3MM Whatman) for molecular genotyping. Haemoglobin concentration was measured using a portable spectrophotometer HemoCue Hb 201+ (HemoCue AB, Ängelholm, Sweden), with a precision of ± 0.3 g/dL.[25] Thick blood smears and filter papers were processed as previously described.[4,24,25]

Parasite clearance half-life was estimated using the parasite clearance estimator developed by World Wide Antimalarial Resistance Network (Oxford, United Kingdom).[28] Only patients with positive blood slides on at least three consecutive sampling time points during the early 72 hours of treatment were included in this analysis. Parasite clearance time was assessed and defined as previously described.[29]

Molecular analysis. Genomic DNA was extracted from dried blood spots collected at baseline and the time of recurrent infection using ABI PRISM® 6100 Nucleic Acid PrepStation (Applied Biosystems, Fresno, CA) for 2006 and 2007–2008 samples,[4,24] whereas a 10% chelex method was used for the 2012–2013 and 2014 samples.[30] The extracted DNA from patients classified as LCF or LPF was genotyped to differentiate recrudescence from reinfection by stepwise genotyping of *P. falciparum* block 3 of merozoite surface protein (msp) 2, block 2 of msp 1, and region II (RII) of glutamate-rich protein,[31] using a nested PCR method described previously.[30] Briefly, the respective initial amplifications were followed by individual nested PCR reactions using family-specific primers for msp 1 (K1, MAD20, and RO33) and msp 2 (FC27 and IC) and seminested for RII of glurp.[31] The amplicons were loaded on agarose gel containing GelRed™ (Biotium, Inc. Hayward, CA), separated by electrophoresis, and then visualized under ultra violet-transillumination (Gel Doc™ (Bio-Rad, Hercules, CA), and sized using Image Laboratory™ software (Bio-Rad). Alleles in each family were considered the same if fragments size were within 20 base pair interval. Patients with recurrent parasitemia, but with negative PCR results were considered to have unresolved PCR-adjusted outcome and were excluded in the final analysis. The cure rate was estimated based on the proportion of patients experiencing therapeutic failure during the 28 days follow-up period; however, the 2006 cohort was excluded from this analysis. Recrudescence was defined as presence of at least one matching allelic band, and reinfection was defined as absence of any matching allelic band at baseline and on the day of parasite recurrence.[31]

Pfmdr1 N86Y and *Pfcrt* K76T were genotyped at baseline using nested PCR followed by restriction fragment length polymorphism using *ApoI* restriction enzyme as previously described.[32]

Study endpoints. The primary outcome was the proportion of patients with PCR-adjusted parasitological cure rate on day 28 from 2007 to 2014. Secondary outcomes included differences in the proportion of patients with day 28 PCR-adjusted parasitological cure rate from 2007 to 2014, proportion of patients with baseline *Pfmdr1* N86 and *Pfcrt* K76, changes in the baseline proportion of patients with *Pfmdr1* N86 and *Pfcrt* K76 from 2006 to 2014, association between the changes in the baseline proportion of patients *Pfmdr1* N86 and *Pfcrt* K76 genotype with the proportional changes in cure rate from 2007 to 2014, median

parasite clearance time and slope half-life in 2006 and 2012–2013 studies, and differences in the median slope half-lives between 2006 and 2012–2013 studies.

Ethical considerations. The studies were approved by the Muhimbili University of Health and Allied Sciences, Tanzania, Food and Drug Authority and the National Institute for Medical Research ethics committees. The molecular work in Sweden was approved by the Regional Ethics Committee, Stockholm. Written informed consent was obtained from all patients and a proxy consent from parents/guardians in patients aged < 18 years, prior to enrolment.

Statistical analysis. Data were double entered in an electronic database and analyzed using SPSS software, version 16 (SPSS Inc., Chicago, IL) and R, version 3.2.3 (R Foundation, Vienna, Austria). Data were analyzed as per protocol. Cure rate end points were analyzed by survival analysis. Changes in cure rate, *Pfmdr1* N86 and *Pfcrt* K76 proportions from 2006 to 2014 were compared using χ^2square tests for trend. Independent χ^2-test or Fisher's exact and *F* test were used to compare the categorical and continuous variables at baseline, respectively. Median slope half-lives were compared between groups using Mann–Whitney test. Analysis of variance was used to compare mean differences between groups. Data were censored at the time of withdrawal for patients lost to follow-up, withdrew consent, and PCR determined reinfection or uncertain PCR outcome. A $P \leq 0.05$ was considered statistically significant.

RESULTS

Patients and baseline characteristics. A total of 590 participants were included in the analysis. Baseline characteristics of the study participants are presented in Table 1. Patients in the 2007–2008 cohort were all under the age of 5 years, a majority were febrile, anemic, and had higher mean parasite density.

Parasite and fever clearance. Parasite and fever clearances are presented in Figure 1. Following treatment, parasite clearance was slower for the 2006 and 2007–2008 cohorts with less than 50% of patients cleared of parasitemia on day 1, but it was rapid for the 2012–2013 and 2014 cohorts. None of the patients had microscopy-determined parasitemia on day 3 in the 2006, 2012–2013, and 2014 cohorts, whereas two patients had parasitemia in the 2007–2008 cohort.

After initiation of medication, fever clearance was slower for the 2007–2008 cohort compared with the 2006, 2012–2013, and 2014 cohorts with 36.6% (64/175) patients having fever on day 1. On days 2 and 3, fever clearance was slower for the 2006 cohort compared with other cohorts. Few patients still had fever on day 3 in all the cohorts.

Parasite clearance half-life. Parasite clearance time and half-lives were evaluated and compared between the 2006 cohort and the subset of 45 patients in the 2012–2013 cohort. There was no data for this analysis for other cohorts, that is, 2007–2008 and 2014. The median parasite clearance time for the 2006 cohort was 36 (interquartile range [IQR], 16–36) hours, and the slope half-life was 5.7 hours (IQR, 2.6–6.8), whereas for the 2012–2013 cohort it was

TABLE 1
Baseline characteristics of the study participants

Characteristic	Year of Study						Test statistics
	2006		2007–2008		2012–2013	2014	
All ages	*N* = 50		*N* = 180		*N* = 140	*N* = 220	
Age (years), mean (SD)	4.3 (2.5)		2.8 (1.3)		4.5 (2.6)	15.0 (15.1)	$F = 68.0$ $P < 0.001$
Sex (female), *n* (%)	31 (62)		93 (51.7)		78 (55.7)	110 (50)	$X^2_3 = 3.0$ $P = 0.398$
Weight (kg), mean (SD)	14.3 (5.5)		12.2 (2.9)		16.8 (5.9)	32.5 (18.5)	$F = 113.3$ $P < 0.001$
Temperature (°C), mean (SD)	38.5 (1.3)		38.6 (1.2)		38.2 (1.3)	38.3 (1.2)	$F = 4.1$ $P = 0.007$
Haemoglobin level (g/dL), mean (SD)	10.1 (1.7)		9.6 (1.9)		10.5 (1.8)	11.3 (1.5)	$F = 30.4$ $P < 0.001$
Parasitemia/µL, geometric mean (95% CI)	21,687 (14,391–32,681)		41,879 (35,950–48,786)		23,768 (18,314–30,846)	8,356 (6,187–11,284)	$F = 30$ $P < 0.001$
Febrile (≥ 37.5°C), *n* (%)	37 (74.0)		147 (82.6)		88 (62.9)	168 (76.4)	$X^2_3 = 16.6$ $P = 0.001$
Anemic (≤ 10 g/dL), *n* (%)	22 (44.0)		103 (57.2)		12 (8.6)	44 (20.0)	$X^2_3 = 108$ $P < 0.001$
Children below 5 years	*N* = 33		*N* = 180		*N* = 76	*N* = 39	
Age (years), mean (SD)	2.7 (1.3)		2.8 (1.3)		2.4 (1.4)	2.7 (1.1)	$F = 0.61$, $P = 0.611$
Sex (female), *n* (%)	18 (54.5)		93 (51.7)		46 (60.5)	17 (43.6)	$X^2 = 0.0$, $P = 0.950$
Weight (kg), mean (SD)	11.1 (2.0)		12.2 (2.9)		15.3 (6.9)	13.2 (2.5)	$F = 11.3$, $p < 0.001$
Temperature (°C), mean (SD)	38.8 (1.4)		38.6 (1.2)		38.2 (1.3)	38.7 (1.2)	$F = 3.2$, $P = 0.024$
Haemoglobin level (g/dL), mean (SD)	9.4 (1.4)		9.6 (1.9)		10.2 (1.9)	10.4 (1.3)	$F = 2.9$, $P = 0.034$
Parasitemia/µL, geometric mean (95% CI)	23,889 (14,451–39,491)		41,879 (35,950–48,786)		49,773 (28,503–86,896)	10,816 (5,076–23,046)	$F = 10.1$, $p < 0.001$
Febrile (≥ 37.5°C), *n* (%)	26 (78.8)		147 (82.6)		46 (60.5)	32 (82.1)	$X^2 = 5.1$, $P = 0.024$
Haemoglobin level < 10 g/dL, *n* (%)	20 (60.6)		103 (57.2)		11 (14.5)	14 (35.9)	$X^2 = 31.6$, $p < 0.001$

CI = Confidence interval; SD = standard deviation.

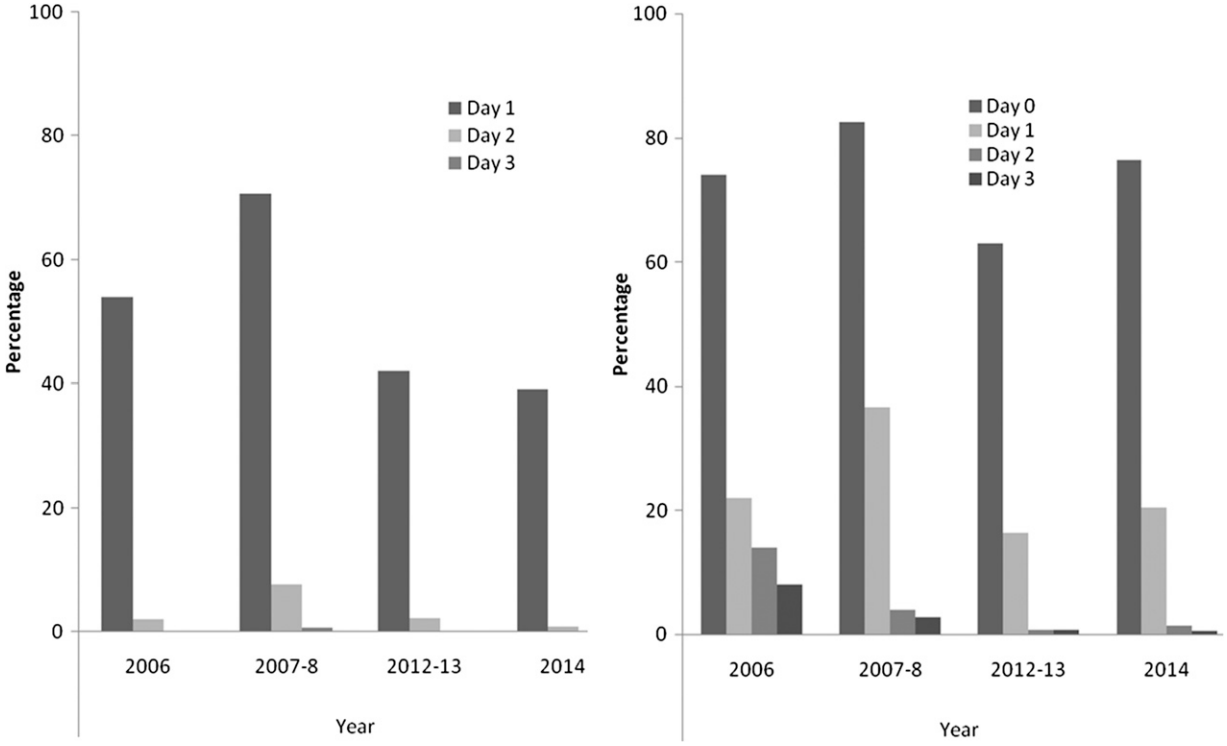

FIGURE 1. Prevalence of patients with parasites (left) and fever (right) following treatment from 2006 to 2014.

24 (IQR, 18–24) hours and the slope half-life was 1.5 hours (IQR, 1.1–2.0), ($P < 0.001$, Mann–Whitney test).

Treatment outcomes. Treatment outcomes from 2007 to 2014 are presented in Table 2. There was no significant change in the trend of cure rate between 2007 and 2014 (χ^2_{trend} test = 0.06, $P = 0.81$), with PCR-adjusted cure rate changing from 168/170 (98.8%) (95% confidence interval [CI], 97.2–100) to 122/127 (96.1%) (95% CI, 92.6-99.5) and to 206/207 (99.5%) (95% CI, 98.6–100) in 2007–2008, 2012–2013, and 2014, respectively.

Prevalence of *Pfmdr1* N86Y and *Pfcrt* K76T. Distribution of baseline *Pfmdr1* N86Y and *Pfcrt* K76T is presented in Figure 2. The prevalence of *Pfmdr1* N86 increased significantly across years from 13/48 (27.1%) in 2006 to 183/213 (85.9%) in 2014 ($\chi^2_{trend} = 92.6$, $P < 0.001$). For *Pfcrt* K76T, the prevalence of *Pfcrt* K76 increased significantly across years from 24/47 (51.1%) in 2006 to 198/205 (96.6%) in 2014 ($\chi^2_{trend} = 73.6$, $P < 0.001$).

***Pfmdr1* N86 and *Pfcrt* K76 prevalence in relation to cure rate.** The association of baseline *Pfmdr1* N86 and *Pfcrt* K76 with the cure rate across years is presented in Figure 3. The figure shows that the cure rate remained high across years regardless of a significant increase in prevalence of *Pfmdr1* N86 and *Pfcrt* K76 between 2007 and 2014.

DISCUSSION

The findings from this study showed that the AL PCR-adjusted cure rate remained high and did not change significantly across years between 2007 and 2014. The observed high cure rate was similar to that reported at baseline,

TABLE 2
Treatment outcome

Outcome	Year of study			χ^2-trend test (*P* value)
	2007–2008 (*N* = 180)	2012–2013 (*N* = 140)	2014 (*N* = 220)	
ETF	1 (0.5)	0	0	1.84 (0.174)
LCF	12 (7.0)	9 (7.1)	8 (3.8)	1.46 (0.227)
LPF	5 (3.0)	3 (2.3)	7 (3.3)	0.02 (0.880)
Unresolved PCR data	1 (0.6)	0	4 (1.9)	3.50 (0.061)
Crude recurrent parasitemia	18 (10.5)	12 (9.4)	15 (7.1)	1.23 (0.267)
PCR determined recrudescence (%)	2 (1.2)	5 (3.9)	1 (0.5)	0.02 (0.900)
PCR determined reinfection	15 (8.8)	7 (5.5)	10 (4.7)	2.81 (0.094)
Lost follow-up	5 (2.8)	12 (8.6)	6 (2.8)	0.19 (0.659)
Withdrawal/Protocol violation	4 (2.3)	1 (0.8)	3 (1.4)	0.19 (0.659)
Day 28 unadjusted ACPR, *n* (%)	153 (89.5)	115 (90.6)	196 (92.9)	1.21 (0.267)
Day 28 PCR-adjusted ACPR, *n* (%)	168 (98.8)	122 (96.1)	206 (99.5)	0.02 (0.900)

ACPR = adequate clinical and parasitological response; CI = confidence interval; ETF = early treatment failure; LCF = late clinical failure; LPF = late parasitological failure N = sample size.

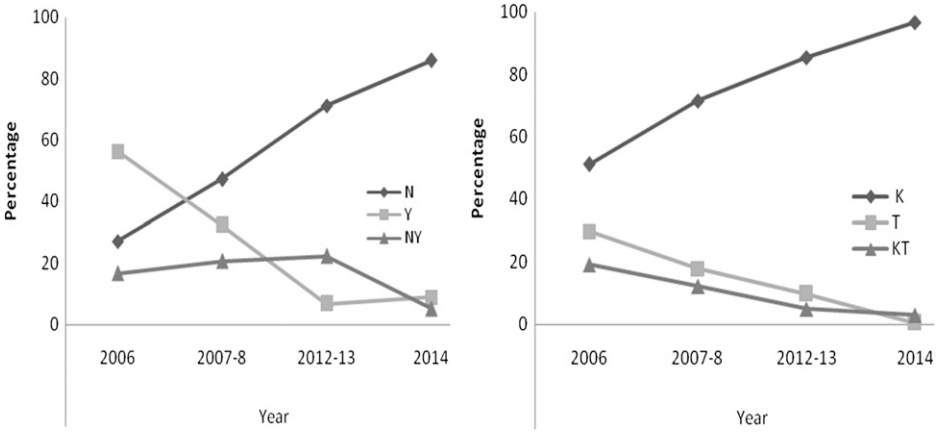

FIGURE 2. Distribution of baseline *Pfmdr1* N86Y (left) and *Pfcrt* K76T (right) from 2006 to 2014. (N = wild type; Y = mutant; NY = mixed infection; K = wild type; T = mutant; KT = mixed infection).

before the implementation of the AL policy,[33,34] and in the follow-up studies conducted in Bagamoyo district and other parts of Tanzania.[4–6,35,36] However, unexpectedly parasite clearance on day 1 was faster for the 2012–2013 and 2014 cohorts, but slower for the 2006 and 2007–2008 cohorts, nonetheless, the clearance was not significantly different between cohorts on days 2 and 3. Furthermore, parasite clearance half-life was rapid for the 2012–2013 cohort (1.5 hours) compared with the 2006 cohort (> 5 hours). Similar rapid parasite clearance as for the 2012–2013 cohort has been reported elsewhere in Africa.[12,37] Nonetheless, it is not well understood why the 2006 cohort had prolonged parasite clearance half-life compared with the 2012–2013 cohort.

This study showed that, there was a significant increase in the prevalence of baseline *Pfmdr1* N86 and *Pfcrt* K76 across years between 2006 and 2014, whereas that of *Pfmdr1* 86Y and *Pfcrt* 76T decreased significantly across years. Similar findings were reported in the same area among recurrent infections[19,20] and in the parasite population after years of wide-scale use of AL between 2004 and 2011.[7] These findings probably suggest that sustained use of AL suppresses the prevalence of the mutant alleles (*Pfmdr1* 86Y and *Pfcrt* 76T), while selecting for the wild type alleles (*Pfmdr1* N86 and *Pfcrt* K76). Furthermore, previous studies have linked selection of *Pfmdr1* N86 and *Pfcrt* K76 with reduced parasite susceptibility against lumefantrine.[13–15,18–20] However, despite the significant increase in the prevalence of *Pfmdr1* N86 and *Pfcrt* K76 in this study, the AL cure rate remained high across years. Nonetheless, the presence of mutations do not always correlate with the measured cure rate.[38,39] The observed high cure rate from this study area and other parts of Africa despite increased selection of *Pfmdr1* N86 and *Pfcrt* K76 across years probably suggest that these genetic markers are not sufficient on their own to give rise to AL resistance.

The main strength of this report is that it has been able to evaluate the AL cure rate before and after the implementation of AL treatment policy in Bagamoyo district. However, in the 2006 cohort the subjects were followed for 3 days only, therefore, it was difficult to predict the drug's cure rate on day 28. Furthermore, there was significant differences in the mean age and baseline parasitemia between the 2014 cohort and other cohorts. Nonetheless, we believe these limitations have not affected the validity of our findings.

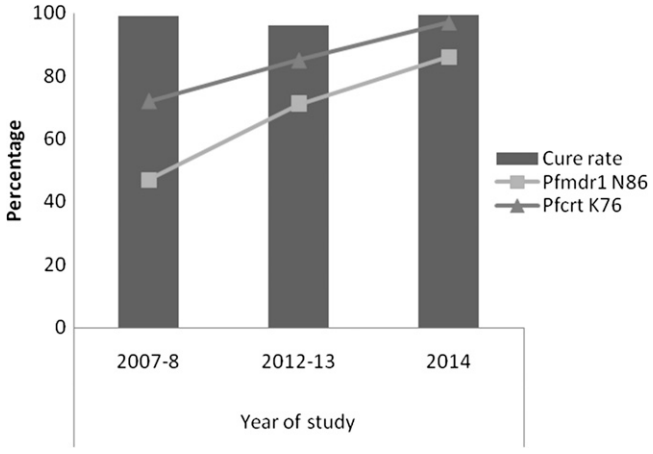

FIGURE 3. Association of baseline *Pfmdr1* N86 and *Pfcrt* K76 with the cure rate across years. N = wild type. K = wild type.

CONCLUSION

The AL cure rate remained high after 8 years of wide-scale use in Bagamoyo district for the treatment of uncomplicated *P. falciparum* malaria despite an increase in the prevalence of pretreatment *Pfmdr1* N86 and *Pfcrt* K76 between 2006 and 2014.

Received October 2, 2016. Accepted for publication March 16, 2017.

Published online May 15, 2017.

Acknowledgment: We thank all the patients and parents/guardians for participating in the study.

Financial support: Sida funded the study.

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