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CLINICAL THERAPEUTICS

Persistence of Residual Submicroscopic *P. falciparum* Parasitemia following Treatment of Artemether-Lumefantrine in Ethio-Sudan Border, Western Ethiopia

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ABSTRACT The emergence of artemisinin-resistant parasites in Africa has had a devastating impact, causing most malaria cases and related deaths reported on the continent. In Ethiopia, artemether-lumefantrine (AL) is the first-line drug for the treatment of uncomplicated falciparum malaria. This study is one of the earliest evaluations of artemether-lumefantrine (AL) efficacy in western Ethiopia, 17 years after the introduction of this drug in the study area. This study aimed at assessing PCR- corrected clinical and parasitological responses at 28 days following AL treatment. Sixty uncomplicated falciparum malaria patients were enrolled, treated with standard doses of AL, and monitored for 28 days with clinical and parasitological assessments from September 15 to December 15, 2020. Microscopy was used for patient recruitment and molecular diagnosis of *P. falciparum* was performed by Var gene acidic terminal sequence (*varATS*) real-time PCR on dried blood spots collected from each patient from day 0 and on follow-up days 1, 2, 3, 7, 14, 21, and 28. *MspI* and *msp2* genotyping was done to confirm occurrence of recrudescence. Data entry and analysis were done by using the WHO-designed Excel spreadsheet and SPSS version 20 for Windows. A *P* value of less or equal to 0.05 was considered significant. From a total of 60 patients enrolled in this efficacy study, 10 were lost to follow-up; the results were analyzed for 50 patients. All the patients were fever-free on day 3. The asexual parasite positivity rate on day 3 was zero. However; 60% of the patients were PCR positive on day 3. PCR positivity on day 3 was more common among patients $<$15 years old as compared with those $\geq$15 years old (AOR = 6.44, P = 0.027). Only two patients met the case definition of treatment failure. These patients were classified as a late clinical failure as they showed symptoms of malaria and asexual stages of the parasite detected by microscopy on day 14 of their follow-ups. Hence, the Kaplan-Meier analysis of PCR- corrected adequate clinical and parasitological response (ACPR) rate of AL among study participants was 96% (95% CI: 84.9–99). In seven patients, the residual submicroscopic parasitemia persists from day 0 to day 28 of the follow-up. In addition, 16% (8/50) of patients were PCR– and then turned PCR+ after day 7 of the follow-up. AL remains efficacious for the treatment of uncomplicated falciparum malaria in the study area. However, the persistence of PCR-detected residual submicroscopic parasitemia following AL might compromise this treatment and need careful monitoring.

KEYWORDS therapeutic efficacy study, uncomplicated falciparum malaria, western Ethiopia

Artemisinin-based combination therapies (ACTs) are currently used as a first-line treatment for uncomplicated falciparum malaria in endemic countries (1). Artemisinin resistance with a clinical phenotype manifested by slow parasites clearance was first reported in western Cambodia and has now emerged or spread to other areas of Southeast Asia (2–4).

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In Africa, it is predicted that the artemisinin-resistant malaria parasites might spread from Asia or originate *de novo*. The existence of artemisinin resistance in Africa is concerning as more than 215 million malaria cases and 38,4000 deaths in 2019 were reported in Africa (5). Although ACT is still effective in Africa, there is increasing concern that antimalarial treatment with ACT would be seriously threatened by the emergence of drug-resistant parasites (6).

Some recent studies in Africa have shown reduced *P. falciparum* susceptibility to ACT and longer parasite clearance time (7–9). In addition, clinically artemisinin-resistant parasites have been reported from Rawada and Uganda (10, 11).

Recent therapeutic efficacy studies (TES) that use quantitative PCR (qPCR) are reporting the persistence of residual submicroscopic parasites after ACT treatment (12–14). Substantial residual submicroscopic parasitemia after microscopically successful artemether-lumefantrine (AL) treatment was reported by qPCR (15).

Recrudescence after ACT treatment might not be the result of inherent parasite resistance (16, 17). Host immunity and pharmacokinetic factors are also determinants of ACT treatment efficacy. Individuals lacking acquired immunity may have higher rates of AL treatment failure (18). In addition, recrudescence was associated with day 7 lumefantrine concentrations in blood (19), concomitant food intake (20), and type of diet taken (21).

Malaria is one of the major health problems in Ethiopia. In 2004, Ethiopia adopted the ACT, artemether-lumefantrine, as the first-line treatment of uncomplicated *P. falciparum* malaria (22). There are a few TES carried out in Ethiopia with a reported greater than 98% PCR corrected cure rate for AL (23–25). However; there is a paucity of reports on the efficacy of AL in western Ethiopia. Therefore, this study was aimed to assess PCR corrected clinical and parasitological response at 28 days following AL treatment and to give evidence of AL treatment outcomes in the treatment of uncomplicated falciparum malaria in the western part of the country that borders Sudan.

RESULTS

Socio-demographic and baseline clinical characteristics of study participants. A total of 60 consented, uncomplicated *P. falciparum* malaria patients were enrolled for the 28 days follow-up. Of these, 16.7% (10/60) patients did not attend the scheduled visits and were found lost to follow-up, and 83.3% of the enrolled patients completed the study (Table 1). No patient was withdrawn from the study.

Thirty-two of the 50 patients were recruited from Sherkole health center. Most of the study participants were Berta in Ethnicity, and 68% (34/50) of them were males. The mean age of study participants was 15.22 years $\pm$ 10.4 SD. The minimum and maximum ages were 2 years and 48 years, respectively. Five of the study participants were under 5 years old, and 56% of the patients were between 5 and 15 years in age (Table 1).

Of patients at enrollment, 40/50 (80%) had fever. The geometric mean of parasitemia at baseline was 21,652 $\pm$ 32740 SD parasites/μL. The minimum and maximum parasite densities were 2,349 and 141,793 parasites/μL, respectively. The first, second, and third quartile ranges of parasitemia at baseline were 6,066.75, 37,633.5, and 62,323.25 parasites/μL, respectively (Table 1).

Clinical and parasitological outcomes of AL treatment. (i) Early and late clinical response. Eight patients had fever on day 1 after they took one dose of AL treatment. On day 2 and day 3, resolution of fever was noted among all patients. For two patients who had fever on day 1, the fever reoccurred on day 7. One patient showed symptoms of malaria (history of fever within 24 h and chills and headache) on day 7. In addition, on day 14 of the follow-up, two patients showed symptoms of malaria (history of fever within 24 h and chills, headache, and back pain).

(ii) Early and late parasitological response. As determined by light microscopy, the prevalence of parasitemia on days 1, 2, and 3 following initiation of AL therapy was 38%, 10%, and 0%, respectively. Parasites were not detected using microscopy, in three patients in which clinical features of malaria were observed on day 7. However, two patients who had the symptoms of malaria on day 14 were microscopically positive

TABLE 1 Socio-demographic and baseline clinical characteristics of uncomplicated *P. falciparum* malaria cases in Sherkole and Horazhab health centers, western Ethiopia, September 15 to December 15, 2021 ($n = 50$)

Variables	Frequency	Percent
Enrolled participants	60	
Lost to follow-up	10	16.7
Complete the follow-up study	50	83.3
Study health centers		
Sherkole health center	32	64
Horazhab health center	18	36
Sex		
Male	34	68
Female	16	32
Age		
<5 yrs	5	10
5–15 yrs	28	56
≥15 yrs	17	34
Ethnicity		
Berta	36	72
Oromo	8	16
Amhara	5	10
Gumz	1	2
Baseline clinical data		
Fever	40	80
Parasitemia:		
<50,000 /μL	28	56
≥50,000 /μL	22	44

during the follow-up period. These two cases had a positive blood slide and repeated full doses of artemether-lumefantrine and were followed up to day 28. Parasites were not seen on the blood smear of the patients on day 21 and day 28 after artemether-lumefantrine retreatment. During the follow-up, gametocytes of *P. falciparum* were detected among 4 patients on day 1; among 2 patients on day 2; in 1 patient on day 3, and in 1 patient on day 28 (Table 2).

The prevalence of parasitemia by PCR on day 1, day 2, and day 3 following initiation of AL therapy was 92%, 78%, and 60%, respectively. Three and two patients in the follow-up who showed clinical features of malaria on day 7 and day14, respectively, were *P. falciparum* positive using PCR. Following AL treatment, residual submicroscopic parasitemia persisted in some patients after completing the full dose of the treatment. Fourteen of 50 (28%) patients tested were parasite positive on day 7 by PCR. Eight patients who were PCR negative after AL treatment became PCR positive during the follow-up. Of those, 2, 3, and 3 patients became PCR positive on day 14, day 21, and

TABLE 2 Parasite positivity using light microscopy and PCR following AL treatment

	Parasite positivity		
Follow-up days	Microscopy	PCR	Gametocytes detected
Day 1	38% (19/50)	92% (46/50)	8% (4/50)
Day 2	10% (5/50)	78% (39/50)	4% (2/50)
Day 3	0	60% (30/50)	2% (1/50)
Day 7	0	28% (14/50)	0
Day 14	4% (2/50)	18% (9/50)	0
Day 21	0	20% (10/50)	0
Day 28	0	20% (10/50)	2% (1/50)

TABLE 3 Socio-demographic and baseline clinical characteristics of patients in relation to PCR positivity on day 3 ($n = 50$)[a]

	Day 3 parasite positivity					
Characteristics	**Positive**	**Negative**	**COR**	***P* value**	**AOR**	***P* value**
Study site						
Sherkole	18	14	1.556	0.472	4.82	0.06
Kurmuc	12	16	1		1	
Ethnicity						
Berta	24	12	2.67	0.129	2.21	0.375
Others	6	8	1		1	
Age						
<15 yrs	24	9	4.89	0.013	6.44	0.027
≥15 yrs	6	11	1		1	
Baseline clinical data						
Fever						
Yes	24	16	1	1	1.5	0.384
No	6	4	1		1	
Parasitemia						
<50,000/μL	15	13	1.857	0.3	1.5	0.562
≥50,000/μL	15	7	1		1	

[a]COR, crude odds ratio; AOR, adjusted odds ratio.

day 28, respectively. In addition, among 7 patients, persistent residual submicroscopic parasitemia was detected from day 0 to day 28 by PCR (Table 2). None of the study participants reported adverse drug reactions to AL.

P. falciparum PCR positivity on day 3 after initiation of AL treatment was not associated with areas of residence, sex, or ethnic group of study participants ($P > 0.05$), but the PCR positivity on day 3 was more among study participants <15 years old as compared with ≥15 years old (AOR = 6.44, $P = 0.027$). Fever and parasitemia at baseline were not associated with PCR positivity on day 3 (Table 3).

Sex, age, ethnicity, and baseline clinical characteristics of study participants were not associated with PCR positivity after day 7 of the follow-up ($P > 0.05$), but there was significant association between area of residence and *P. falciparum* PCR positivity after day 7 of the follow-up (AOR = 0.05, $P = 0.002$) (Table 4).

Quantification cycle value analysis of the *P. falciparum* on day 0 and the follow-up days of the seven patients with persistent PCR positivity indicated the rapid reduction of the parasite density following treatment. However, after day 7 of the treatment the residual submicroscopic parasitemia level remained constant and persisted in patients, as shown in (Fig. 1).

(iii) PCR-corrected assessment of AL treatment outcomes. The two patients that showed recurrent malaria on day 14 were confirmed as recrudescence by *msp1* and *msp2* genotyping of day 0 and day 14 samples (Table 5). Thus, PCR confirmed that AL late clinical failure (LCF) occurred in two patients. Kaplan-Meier estimate of the day 28 efficacy rate of AL among study participants was 96% (95% CI: 84.9–99) with PCR corrected (Fig. S1 in the supplemental material).

DISCUSSION

WHO advocates increased monitoring and surveillance of ACT efficacy against malaria to identify and contain artemisinin resistance (26). Malaria-endemic areas in the Asossa zone are not represented in current surveillance of ACT- resistance malaria in Ethiopia; therapeutic efficacy of AL was not done in this area for more than a decade.

In this study, all patients were fever-free on day 3. Nonetheless, three patients showed clinical features of malaria on day 7. In these patients, the presence of the *P. falciparum* parasite was confirmed using PCR although this was not detected by microscopy. In addition, on day 14 of the follow-up, two patients showed symptoms of

TABLE 4 *P. falciparum* PCR positivity after day 7 in association with socio-demographic and baseline clinical characteristics of patients[a]

	PCR positivity					
Characteristics	**PCR+**	**PCR–**	**COR**	***P* value**	**AOR**	***P* value**
Study site						
Sherkole	4	28	0.091	0.001	0.05	0.002
Kurmuk	11	7	1	1	11	
Sex						
Male	9	25	0.6	0.43	0.861	0.852
Female	6	10	1		1	
Ethnicity						
Berta	11	25	0.91	0.95	0.547	0.51
Others	4	10	1		1	
Age						
<15 yrs	10	23	0.96	0.891	0.314	0.28
15 yrs	5	12	1		1	
Baseline clinical data						
Fever						
Yes	12	28	1	1	0.638	0.681
No	3	7	1		1	
Parasitemia						
<50,000/μL	10	18	1.9	0.323	1.12	0.83
≥50,000/μL	5	17	1		1	

[a]COR, crude odds ratio; AOR, adjusted odds ratio.

malaria (history of fever within 24 h and chills, headache, and back pain). In these patients, the presence of the parasite was confirmed using both light microscopy and PCR. These findings indicated that AL treatment effectively resolved malaria symptoms on day 3; few patients developed clinical malaria during the follow-up period.

The parasite positivity rate on day 3 by light microscopy was zero. Thus, on day 3 all study patients cleared the parasites based on microscopy. However, 60% of patients were PCR positive on day 3. This PCR positivity after artemether-lumefantrine therapy might be due to residual DNA and/or gametocytes in the absence of viable parasites, indicating that PCR might overestimate parasite prevalence on day 3 after treatment. This posttreatment residual submicroscopic parasite prevalence was comparable with the residual parasitemia on day 3 (68.5%) reported from Faladje, Mali (8). The rate was also higher than 17.7% reported in another study in Mali and Burkina Faso (27).

PCR positivity on day 3 after initiation of the drug was more common in the age group <15 years compared to study participants ≥15 years old (AOR = 6.44,

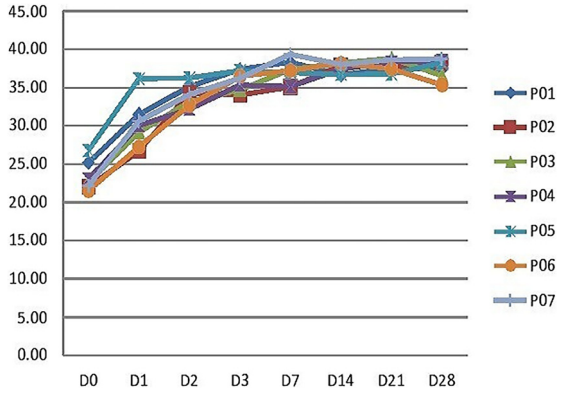

FIG 1 Quantification cycle (Cq) value of *P. falciparum* on day 0 and follow-up days of seven patients with persistent PCR positivity as detected by real-time PCR.

TABLE 5 A measured band lengths of *msp1* and *msp2 of* the two patients with recrudescences on day 0 and day of recurrency

		Band length of the Loci in bp	
Patients with treatment failure	**Genes**	**Day 0**	**Day 14**
Patient 1	*msp1*	160	159
	msp2	202, 328, 337	202, 328
Patient 2	*msp1*	186, 248	186, 249
	msp2	222,342	221, 341

$P = 0.027$). The association between increasing age and early parasitological clearance has been previously reported (28–30). In high malaria transmission areas, the chance of developing acquired immunity to malaria increases as individuals get older. Acquired immunity is lower in younger individuals, and it has a pivotal role in modulating early parasitological response to treatment with ACTs (29, 31). PCR positivity on day 3 was not associated with baseline parasite density. This finding contrasted with previous findings in Mali and Burkina Faso (27) and Tanzania (32) and could be related to host or parasite factors.

Efficacy data from the current study showed a high cure rate of AL. The PCR-corrected adequate clinical and parasitological response on day 28 was 96%. Two patients (4%) were classified as a late clinical failure as they showed symptoms of malaria and parasite positivity on day 14 detected in microscopy. This study indicated that AL remains highly efficacious for the treatment of uncomplicated *P. falciparum* infection in the study site. This treatment success was comparable to previous studies done in Ethiopia (23, 33). In neighboring Sudan, a similar high treatment success rate of ACT was reported from a meta-analysis of 20 studies (34).

The persistence of PCR positivity following AL in this study was similar to those in Kenya that reported 37.1% residual parasitemia on day 7 (15), Uganda with 39·9% submicroscopic parasitemia persistence in children on day 17 (14), and Sumatra that reported 39% (35). The finding was also similar to another study that was done in Uganda that reported more than 25% of patients had circulating ring-stage parasites by qRT-PCR at least 14 days postinitiation of ACT or ACT-primaquine (13).

Parasite positivity in PCR after day 7 was more in the Kurmuk district as compared with the Sherkole district (AOR = 0.05, $P = 0.002$). Persistence of the residual submicroscopic parasitemia in some patients might relate to inadequate artemether-lumefantrine levels in the blood of study participants following treatment. The role of the lumefantrine partner drug is to eliminate the residual parasites and cure the infection (36). Low lumefantrine concentration on day 7 was associated with *P. falciparum* recrudescence (19). Concomitant food consumption and the type of food taken might affect the absorption of lumefantrine among study participants (20, 21).

Although the treatment is effective in the study area, it is crucial to further assess factors that might relate to PCR positivity following AL treatment. The persistence of positive PCR after curative treatment of AL might be related to several weeks' persistence of the remaining parasite DNA and gametocytes after treatment without evidence of viable asexual parasites (37, 38). This finding highlights the need of distinguishing active infections from dead pathogens or their debris (39) and submicroscopic gametocytemia (40).

Some patients remained PCR positive during the entire study period and might be reservoir hosts after the 28 days of the follow-up. In these patients, the residual submicroscopic parasitemia persists after AL treatment. This may contribute to the onward transmission of malaria among the surrounding human population. Residual *P. falciparum* parasitemia after ACT is associated with increased transmission to mosquitoes (41); 1. 88% of mosquitoes became infected after feeding on blood from AL-treated children (42). Asymptomatic recrudescence may be important for the spread of drug-resistant malaria (43, 44).

The persistence of the residual submicroscopic parasitemia in the current study indicated the role of microscopy and PCR in reporting the parasites following treatment with

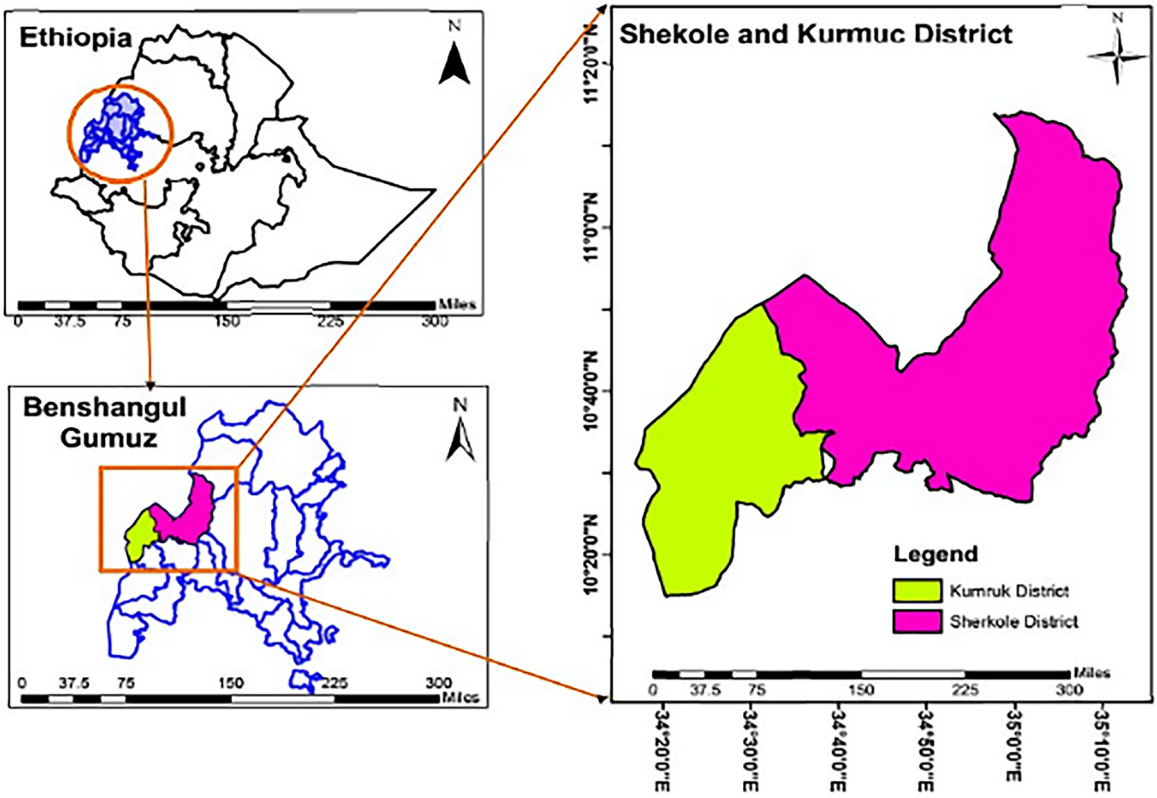

FIG 2 Study area map, Sherkole and Kurmuk districts in the Asossa zone. Benishangul-Gumuz Regional State, western Ethiopia.

AL. Microscopy alone might not indicate the actual prevalence of parasitemia following initiation of ACT therapy. Thus, therapeutic efficacy studies should consider using ultrasensitive detection of *P. falciparum* to confirm the presence of low-density parasitemia and/or residual DNA in the absence of viable parasites or gametocytes following treatment of ACTs (45).

Conclusion. Artemether-lumefantrine remains efficacious for the treatment of uncomplicated *P. falciparum* in the study area. However, in some patients, there was residual submicroscopic parasitemia after AL treatment. The persistence of PCR positivity and reappearance of PCR detectable parasites following AL treatment has public health implications for continued malaria transmission and may be important for the spread of drug-resistant malaria. There is a need to assess factors that contribute to the PCR positivity of the parasite that might compromise the treatment.

Limitations of the study. Small sample size, lack of direct observation of the second dose of the drug, and absence of hemoglobin assessment were the limitations of this study.

MATERIALS AND METHODS

Study site and period. This study was conducted in Sherkole and Horazhab health centers close to the Ethio–Sudan border, located at 10.599 and 34.7803 and 10.56 and 34.2967 latitude and longitude, respectively. The two health centers are found in the Asossa zone in the Benishangul-Gumuz Region of Ethiopia. Sherkole Health Centre is located in the Sherkole district, which is bordered by Sudan in the north (Fig. 2). This district has a refugee camp that houses more than 14,431 Sudanese and South Sudanese (46). The town of Sherkole is about 98 km west of the town of Asossa. Horazhab health center is located in the Kurmuk district, and it is bordered by Sudan in the north and west. It is about 90 km from the town of Asossa (Fig. 2). This study was conducted during the high malaria transmission season from September to December 2020.

Study design. The study was a prospective study design to assess the clinical and parasitological efficacy of AL for the treatment of uncomplicated falciparum malaria.

Sample size. The sample size calculation was based on an assumed 97.01% therapeutic efficacy of AL in Ethiopia (33), a 95% confidence level, and 5% precision. Accordingly, the calculated sample size was 45. An additional 20% nonresponse rate was considered, so the sample size became 54.

Study population. Patients with uncomplicated *P. falciparum* malaria attending the study health centers who consented to this study were enrolled if they were more than 2 years of age, had a fever (axillary temperature ≥37.5°C), and/or had a history of fever in the last 24 h, mono-infection with *P. falciparum*, and parasitemia of 2,000 to 200,000 asexual parasites/μL by microscopy. Patients who had received antimalarial drugs 6 days before enrollment were excluded from the study. Out of 50 patients who fulfilled the inclusion criteria and enrolled in the study, 32 and 18 patients were recruited from the Sherkole and Horazhab health centers, respectively. Once eligible patients were enrolled at the two study sites, they were treated with standard doses of AL and monitored for 28 days with clinical and parasitological assessments. Patients' clinical and parasitological assessments were done on day 0 and follow-up days 1, 2, 3, 7, 14, 21, and 28 based on the WHO template protocol for therapeutic efficacy studies (47).

Treatment of patients. Artemether 20 mg + Lumefantrine 120 mg tablets (Ajanta Pharma Ltd, India) was administered twice daily for 3 days according to the weight of study participants: one tablet for ≥5 kg to <15 kg, two tablets for 15 kg to <25 kg, three tablets for 25 kg to < 35 kg, and four tablets for ≥35 kg. The first daily dose of the drug was directly observed at the study health centers. However, the second daily dose of the drug, taken at home, was not supervised. Then, after each dose, patients were observed for 30 min, and the dose was readministered if vomiting occurred. Adverse events were assessed and recorded during each visit of the patients.

Laboratory diagnosis of *P. falciparum*. (i) Microscopy. Microscopy was performed from finger-prick blood collected during days 0, 1, 2, 3, 7, 14, 21, and 28. In addition, dried blood spots (DBS) on filter paper (Whatman No. 1001 320, International Ltd., Maidstone, England) were collected from finger-prick during each subsequent visit of the patients for molecular analysis of the parasite. The DBS was kept in plastic bags with desiccants.

Two blood slides were prepared during each subsequent visit, and both slides were stained with 10% Giemsa for 10–15 min. One of the stained slides was examined at the study health centers to determine the infection status of the patients and to estimate the parasite density. The second stained slide was kept for reading by a second microscopist at the Adama health center. If there was any discrepancy in the parasitemia report between the first and second reader, a third microscopist would read the slides.

Thick and thin Giemsa-stained blood films for parasite counts and species identification were examined at the screening on day 0 to confirm adherence to the inclusion criteria. Thick blood films were examined on days 1, 2, 3, 7, 14, 21, and 28 to quantify malaria parasites. Parasitemia was measured by counting the number of asexual parasites against 200 leucocytes in thick blood films. Parasites per microliter of blood were determined assuming 8,000 white blood cells (WBCs) per microliter of blood.

(ii) Molecular diagnosis. The molecular diagnosis of the parasites was done at Medical Research Council Unit The Gambia at the London School of Hygiene and Tropical Medicine. It was performed on DNA extracted from DBS collected from each patient from enrollment (day 0) to days 1, 2, 3, 7, 14, 21, and 28 using the Chelex DNA extraction protocol as earlier described (48).

P. falciparum detection was performed by var gene acidic terminal sequence (*varATS*) real-time PCR using the Bio-Rad CFX96 real-time PCR machine as previously described (45). This assay had a limit of detection of 0.06 to 0.15 parasites/μL blood and was 10× more sensitive than standard 18S rRNA qPCR (45). Quantification cycle (C_q) values less than 40 were indicative of the *varATS* amplification in the samples. Discrimination of recrudescence from new infections was determined by comparing merozoite surface protein 1 and 2 alleles (*msp1* and *msp2*) of samples collected at baseline with those collected on the day of recurrent infection observed after day 7.

MspI and *msp2* genomic DNA of the parasite was amplified by multiplex primary and nested PCR using allelic specific primers as per published protocol (49). Allelic variants of *msp1* (K1, MAD 20, and RO33) and *msp2* (FC27 and 3D7) were detected by allelic family-specific nested PCR amplification. Genotypes were distinguished using their fluorescent dye (indicating the allelic family) and by their size, which was determined by Qiaxcel software.

Data analysis. Data entry and analysis was done by using the WHO-designed Excel spreadsheet and SPSS 20.0 statistical software package (SPSS, Inc., Chicago, USA). AL treatment outcomes were classified based on parasitological and clinical outcomes assessments as recommended by WHO (50). Descriptive statistics were reported. Binary logistic regression analysis was done to determine factors associated with parasite PCR positivity during the follow-up. A *P* value of less or equal to 0.05 was considered significant.

Ethical consideration. Ethical approval was obtained from the Ethiopian National Ethic review committee, Addis Ababa, Ethiopia (No. MoSHE 04/246/66) and Aklilu Lemma Institute of Pathobiology, Institutional Review Board, Addis Ababa University (No. ALIPB IRB/19/2012/20). Permission to conduct the study at the health facilities was sought from the relevant regional and district health authorities. Written informed consent was obtained from adult study participants and a parent or guardian of a child. Written informed assent was also taken from children.

Data availability. The data sets and analyzed result of the study are available on the corresponding author and can be obtained on reasonable request.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.04 MB.

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We declare that we have no competing interests.

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