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Efficacy of artesunate + sulphadoxine/pyrimethamine and artemether + lumefantrine and *dhfr* and *dhps* mutations in Somalia: evidence for updating the malaria treatment policy

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Abstract

OBJECTIVE To determine the therapeutic efficacy of artesunate + sulphadoxine/pyrimethamine (AS + SP) and artemether + lumefantrine (AL), and to investigate the presence of molecular mutations associated with resistance, to inform national malaria treatment policy.

METHODS One-arm prospective studies were conducted in three study sites in Somalia in 2013 and 2015 to evaluate the efficacy of AS + SP and AL among patients with uncomplicated falciparum malaria. Outcomes included clinical and parasitological response over 28 days, and the presence of dihydrofolate reductase (*dfhr*) and dihydropteroate synthase (*dhps*) and mutations.

RESULTS Among patients treated with AS + SP, the PCR-corrected treatment failure rate was 12.3%. The majority of patients (89%) carried either the quintuple mutations (51I/108N + 437G/540E/581G or 51I/59R/108N + 437G/540E) or the quadruple mutation (51I/108N + 437G/540E). All patients who failed treatment with AS + SP carried the quintuple mutation (51I/108N + 437G/540E/581G). In the studies of AL, the PCR-corrected treatment failure rate was <6%. All patients in both treatment groups cleared their parasitaemia by day 3.

CONCLUSIONS The findings demonstrate a failing first-line treatment (AS + SP), with a failure rate above the threshold (10%) for policy change, and a high prevalence of quintuple mutations. In contrast, AL was highly efficacious. Based on these findings and the results from a previous AS + SP study, AL was selected to replace AS + SP as the first-line treatment for uncomplicated malaria in Somalia in 2016. Dihydroartemisinin + piperaquine (DHA + PPQ) has been recommended as the second-line treatment. Routine monitoring of recommended ACTs should continue to inform treatment policy.

keywords artesunate + sulphadoxine/pyrimethamine, artemether + lumefantrine, dihydroartemisinin + piperaquine, efficacy, *Plasmodium falciparum*, Somalia

Introduction

The emergence and spread of drug-resistant malaria remains a challenge to malaria control. The development of *Plasmodium falciparum* resistance to safe and cost-effective antimalarial drugs such as chloroquine and sulphadoxine + pyrimethamine (SP) [1] has been associated with an increase in malaria mortality [2, 3]. In 2001, to address the emergence of drug resistance, WHO recommended the use of artemisinin-based combination therapy (ACT) for the treatment of uncomplicated malaria [4]. With an effective ACT, the artemisinin component rapidly clears parasites from the blood, and the longer-acting partner drug clears the remaining parasites. In

accordance with WHO recommendations, Somalia adopted AS + SP as the first-line treatment for uncomplicated malaria in 2006 [5], and AL as the recommended second-line drug in 2011. However, in 2011, studies conducted in Somalia's southern zone detected a high proportion of treatment failures (22%) with AS + SP in the study site Jamame, and high levels of *dhfr/dhps* quadruple and quintuple mutations in the study sites Jamame and Jowhar [6].

Resistance to SP has been attributed to point mutations in the parasite genes encoding their target enzymes, dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*). These mutations accumulate at several sites in the *dhfr* and *dhps* genes [7]. The quintuple mutant, a combination of the *dhfr* triple mutant (51I + 59R + 108N) and the *dhps* double mutant (437G + 540E), is found throughout sub-Saharan Africa and is associated with SP treatment failure [7–10].

In keeping with WHO's recommendation to monitor the efficacy of the first- and second-line treatment in malaria-endemic countries [11], we assessed the therapeutic efficacy and safety of AL and AS + SP, and the presence of *dhfr and dhps* mutations. In 2013, in anticipation of the need for an effective replacement of the first-line treatment, therapeutic efficacy studies of AL were conducted in two study sites (Janale and Jowhar). Subsequently, in 2015, to evaluate the geographical extent of AS + SP treatment failure, as well as the efficacy and safety of AL, studies were conducted in a new study site, Bosaso, located in the north-east (Puntland). The findings from these and previous studies [6] provided the evidence needed for the National Malaria Control Program of Somalia to make an informed decision to change the malaria treatment policy in 2016.

Methods

Study design and sites

Open-label, one-arm prospective studies were conducted to evaluate the therapeutic efficacy of AS + SP and AL for the treatment of uncomplicated falciparum malaria. The studies were conducted during the peak malaria transmission period in Janale and Jowhar (southern zone), and in Bosaso (Puntland) (Figure 1). The study sites represent both moderate-to-high transmission areas (Janale and Jowhar), where most malaria cases occur, and low transmission areas (Bosaso). Studies were conducted from August to October 2013 (AL in Janale and Jowhar) and from February to March 2015 (AL and AS + SP in Bosaso).

Study population

Patients were enrolled in the study if they were between 6 months and 60 years of age, had a fever (≥37.5 °C) or history of fever in the previous 24 h, had *Plasmodium falciparum* mono-infection with a parasite density of 500–200 000 asexual parasites/μl and provided consent (or consent was provided on behalf of the patient by the parent/guardian) to participate in the study. Patients were excluded if they had danger signs or severe malaria as per WHO classification [12], falciparum mixed infection, infection with a non-falciparum species, a febrile illness other than malaria, severe malnutrition in children (mid-upper arm circumference <110 mm), regular medication which might have interfered with antimalarial drug pharmacokinetics (ketoconazole lopinavir + ritonavir, nevirapine, efavirenz, etravirine and rifampicin, carbamazepine, phenytoin, metoprolol, imipramine, amitriptyline, clomipramine, trimethoprim or trimethoprim + sulphamethazole), history of contraindications, history of hypersensitivity reactions to the test drugs or pregnant. As it is culturally unacceptable in Somalia to test female minors and unmarried women for pregnancy for the purposes of scientific research, this group was ineligible for inclusion in the study.

Treatment and patient follow-up

For the AS + SP study, patients were given a daily dose of artesunate (4 mg/kg body weight over 3 days), co-administered with a single dose of 25 mg sulphadoxine/1.25 mg pyrimethamine per kg body weight, under direct supervision. Patients participating in the AL studies received six doses twice daily over three days. The treatment was given according to the recommended weight bands as follows: one tablet for patients weighing 5–14 kg; two tablets for patients 15–24 kg; three tablets for patients 25–34 kg; and four tablets for patients weighing ≥35 kg. If a patient vomited within 30 min of treatment intake, a full dose was re-administered. WHO provided both study medicines. Patients who failed treatment with AS + SP were treated with AL twice daily for 3 days. Those who failed treatment with AL were given quinine 10 mg/kg body weight, three times a day for 7 days. Adverse events and severe adverse events were monitored clinically at enrolment and at each follow-up visit. An adverse event and a severe adverse event were defined according to the WHO protocol for monitoring therapeutic efficacy of antimalarial medicines [13].

The study method was based on the WHO standard protocol for monitoring antimalarial drug efficacy [13]. Clinical assessment was performed at enrolment (day 0)

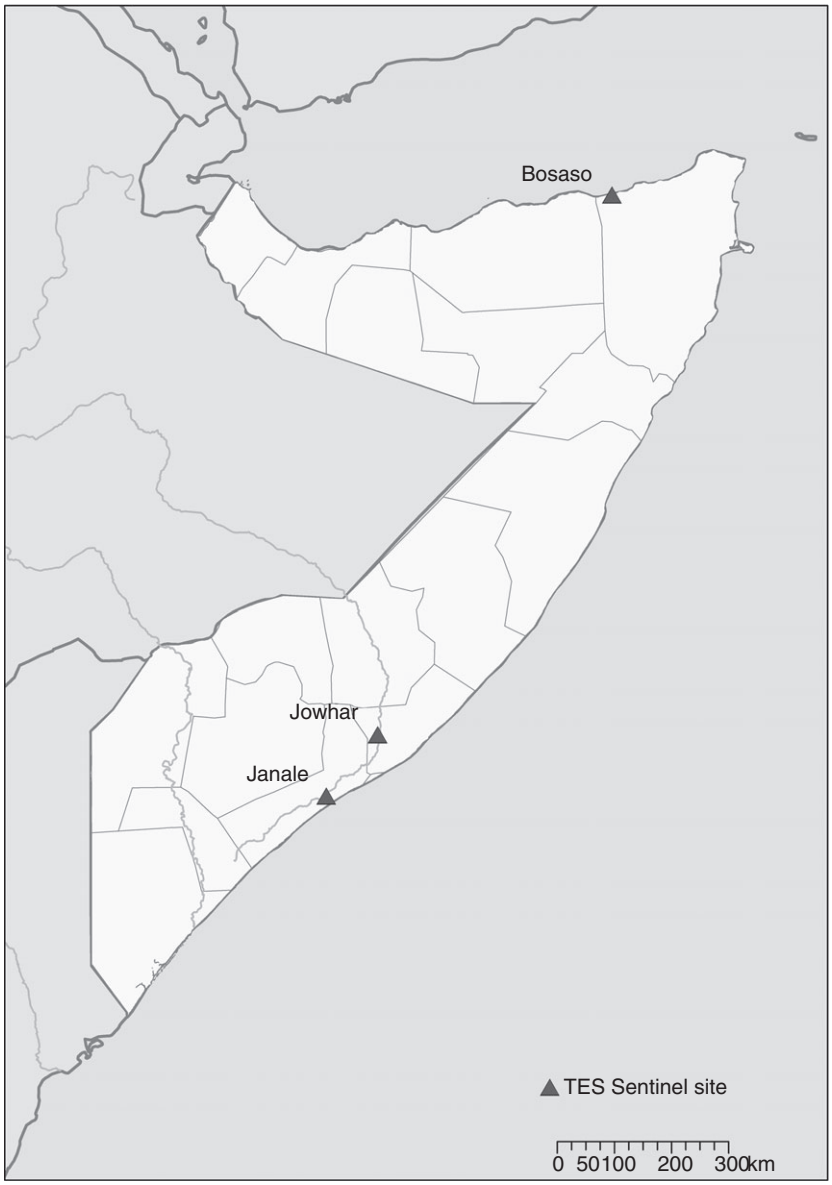

Figure 1 Map of Somalia study sites (2013–15)

and on days 1, 2, 3, 7, 14, 21 and 28, and on any other day if new or recurrent symptoms occurred. Blood slides were taken on the same days as listed above, except day 1. Parasite density was determined assuming a white blood cell count of 8000/μl. A smear was declared negative if no parasites were seen after 1000 white blood cells were counted. Thin blood smears were used for *Plasmodium* species detection. Parasitaemia was determined by two independent microscopists; parasite density was calculated by averaging the two counts. Blood smears with discordant results (differences between the two microscopists in species diagnosis, or where there was a difference in parasite density of >50% or the presence of parasites) were re-examined by a third, independent microscopist, and parasite density was calculated by averaging the two closest counts. Treatment outcomes were classified as early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF) and adequate clinical and parasitological response (ACPR). Exclusion criteria after enrolment included the following: use of antimalarial drugs outside the study protocol, presence of concomitant infection (measles, acute lower

respiratory tract infection, severe diarrhoea with dehydration or other known underlying chronic or severe disease, as assessed through history taking and physical examination), withdrawal of consent, reinfection or lost to follow-up. The proportion of cases positive on day 3 was also recorded.

Parasite genotyping and molecular markers for SP resistance

Paired filter papers were used for parasite DNA extraction and genotyping in cases of parasite recurrence. DNA from day 0 (before treatment) and the day of parasite recurrence was isolated from dried blood spots using a DNA isolation kit, QIAamp, according to the manufacturer's instructions (Qiagen, Valencia, CA, USA) for molecular genotyping. Spots were tested using nested polymerase chain reaction (PCR) targeting polymorphic variant genes *msp1*, *msp2* and *glurp* to distinguish recrudescence (true treatment failures) from new infections, by the observation of band shifts after agarose gel electrophoresis. For PCR-uncorrected data, all patients with parasite recurrence are presented as treatment failures. For PCR-corrected data, only those patients with recrudescence are considered treatment failures. Patients with parasite recurrence, which is classified as a new infection or unknown, are excluded from the analysis.

Using nested PCR, DNA extracted from blood spots on day 0 from the AS + SP group was analysed to detect *dhfr* and *dhps* mutations. Then positive PCR products were subjected to DNA sequencing using BigDye terminator chemistry (Applied Biosystems, Foster City, CA) on a 3130 xl Genetic Analyzer. DNA sequences were assembled, and mutations were verified by inspection of both forward and reverse strands using BioEdit version 7.2.5. Mutations at codons 51, 59, 108, 164 for *dhfr* and codons 436, 437, 540, 581, 613 for *dhps* were assessed.

Sample size and data analysis

Sample size was estimated with a confidence level of 95%, a precision level of 5% and an expected treatment failure rate of 5%. The target sample size was 73. An additional 15 patients (20%) were added to replace patients lost to follow-up or withdrawn.

Data entry was performed using a Microsoft® Excel program developed by WHO (http://www.who.int/malaria/publications/atoz/9789241597531/en/), which requires double data entry and has built-in data validation. Statistical data analysis was conducted using Stata/IC 11.0 (Stata Corporation, College Station, Texas). Baseline patient characteristics were compared across the three study sites. Patients were excluded from the per-protocol analysis of treatment outcomes if they were lost to follow-up, had reinfections or had unknown PCR. In the Kaplan–Meier analysis, all patients were included until the day of withdrawal from the study. The chi-square test was used to compare categorical data. Fisher's exact test was used where cell counts were less than 5. Differences in the mean were evaluated using the t-test. Confidence intervals were calculated for binomial proportions.

Ethical considerations

After reading the study information to the patients, written informed consent was obtained from adult patients or parents/guardians of children under 18 years of age. If the adult patient or parent/guardian was illiterate, an accompanying relative/friend served as a witness. Informed assent was obtained from children aged 12 years and above. The studies were approved of by the Ministry of Health of the Federal Government, the Ministry of Health of Puntland and the WHO Research Ethics Review Committee. Community leaders were informed about the study objectives and procedures and provided their permission.

Results

Some differences in patient baseline characteristics were observed between study sites (Table 1). Patients in Bosaso had more males in both study groups than those in Janale ($P < 0.0001$) and Jowhar ($P < 0.0001$). The mean age of Bosaso patients in the AL study was significantly higher than that of patients in Janale ($P < 0.0001$) and Jowhar ($P < 0.0001$). Baseline geometric mean parasite density was significantly lower among patients in Janale than in Bosaso ($P < 0.001$) and Jowhar ($P < 0.001$). At the time of enrolment, all patients had a history of fever in the prior 24 h.

For the study of AS + SP in Bosaso, the PCR-corrected treatment failure rate was 12.3% (95% CI: 6.1–21.5). There was no difference in the baseline mean age ($P = 0.3$) or geometric mean parasitaemia on day 0 ($P = 0.76$) between patients with treatment failure (LCF + LPF) and those with ACPR. Patients were excluded from the PCR-corrected analysis after withdrawal of consent (1), lost to follow-up (4) and reinfection (4).

For the studies of AL, the PCR-corrected treatment failure rate was less than 6% in all three study sites. Patients were excluded from the PCR-corrected analysis due to lost to follow-up (3) and reinfection (3). All patients in both treatment groups were parasite-free on

Table 1 Patient baseline characteristics and treatment outcomes

	Bosaso (2015) AS + SP*	Bosaso (2015) AL†	Janale (2013) AL†	Jowhar (2013) AL†
N	90 (100)	90 (100)	94 (100)	100 (100)
Males, *N* (%)	72 (80)	79 (87.8)	48 (51.1)	63 (63.0)
Age group, *N* (%)				
<5 years	13 (14.4)	9 (10.0)	27 (28.7)	21 (21)
5 to <15 years	39 (43.3)	33 (36.7)	62 (66)	71 (71)
Adults (15 years and older)	38 (42.2)	48 (53.3)	5 (5.3)	8 (8)
Mean age (years) (SD)‡	17.3 (13.8)	20.9 (15.3)	7.5 (6.8)	8.4 (5.7)
Range (min-max)	1–55	1–60	1–58	2–36
Mean temperature (°C), day 0 (SD)‡	37.8 (0.4)	38.4 (0.5)	37.8 (0.3)	37.7 (0.2)
Geometric mean parasitaemia, day 0 (per µl)	7843	10 349	5503	13 291
Range (min–max)	(819–150 167)	(8089–13 239)	(4358–6949)	(10 757–16 422)
PCR-uncorrected treatment outcomes, day 28	*N* (%)	*N* (%)	*N* (%)	*N* (%)
Late clinical failure	9 (10.6)	5 (5.8)	0 (0.0)	0 (0.0)
Late parasitological failure	5 (5.9)	0 (0.0)	0 (0.0)	1 (1.0)
ACPR§	71 (83.5)	82 (94.2)	94 (100)	99 (99.0)
Total patients per protocol	85 (100)	87 (100)	94 (100)	100 (100)
Withdrawal/lost to follow-up	5 (5.6)	3 (3.3)	0 (0.0)	0 (0.0)
PCR-corrected treatment outcomes, day 28				
Late clinical failure	6 (7.4)	2 (2.4)	0 (0.0)	0 (0.0)
Late parasitological failure	4 (4.9)	0 (0)	0 (0.0)	1 (1.0)
Adequate clinical and parasitological response	71 (87.7)	82 (97.6)	94 (100)	99 (99.0)
Total patients per protocol	81 (100)	84 (100)	94 (100)	100 (100)
Withdrawal/lost to follow-up/reinfections	9 (10)	6 (6.6)	0 (0.0)	0 (0.0)
Cumulative incidence of treatment failure (Kaplan–Meier)	10 (12.1)	2 (2.3)	0 (0.0)	1 (1.0)

*Artesunate + sulphadoxine-pyrimethamine.
†Artemether + lumefantrine.
‡Standard deviation.

day 3. All therapeutic regimens of the study medicines were well tolerated and safe.

The presence of *dhfr* and *dhps* genotypes was assessed on day 0 for the AS + SP study in Bosaso ($n = 90$). All samples carried the *dhfr* S108N and N51I mutant alleles, and 22% carried the *dhfr* C59R mutation in addition Table 2. No *dhfr* 164 mutation was detected. For *dhps,* most of the samples were found to carry either the *dhps* double (437G/540E) or triple (437G/540E/581G) mutation. There were no *dhps* mutations found at codons 436 or 613. For the *dhfr/dhps* combined mutations, 89% of the infections carried the quadruple (51I/108N + 437G/540E) or quintuple (51I/108N + 437G/540E/581G or 51I/59R/108N + 437G/540E) mutation Table 2. All AS + SP treatment failures carried the quintuple (51I/108N + 437G/540E/581G) mutation. The majority (70.4%) of patients with ACPR carried the quintuple mutations. There was no difference in the mean age of patients with treatment failure (17.4 years, 95% CI 8.7–26.1) and those with ACPR who were carrying quintuple mutations (16.2 years, 95% CI 12.2–20.2), quadruple (16.0 years, 95% CI 10.7–19.3) or *dhfr* triple + *dhps* wild (14.6 years, 95% CI 5.5–23.6).

Discussion

We are reporting a PCR-corrected AS + SP treatment failure rate of 12% in Bosaso, located in the north-eastern zone. Given that a high treatment failure rate (22%) with AS + SP was previously reported from Jamame in the southern zone of the country [6], *P. falciparum* resistance to this ACT is likely to be widespread. The treatment failure rate exceeds the WHO-recommended 10% threshold for changing antimalarial treatment policy [11]. Jamame and Janale are located in moderate-to-high transmission areas along Shabelle and Juba rivers, respectively, while transmission is low in Bosaso. These differences in transmission levels might explain the differences in age profiles of the study populations in these sites.

The treatment failure rates reported in the current and previous study [6] are most likely due to resistance to SP.

Table 2 Prevalence of *dhfr* and *dhps* genotypes and their association with PCR-corrected treatment outcome in the AS + SP study conducted in Bosaso, Somalia, 2015

	dhfr and *dhps* genotypes	Excluded*	AS + SP† treatment outcome	
Mutations	*n* (%)	*n*	TF‡ *n* (%)	ACPR§ *n* (%)
dhfr (*n* = 90)				
Double 51I/108N	70 (77.8)	7	10 (15.9)	53 (74.6)
Triple 51I/59R/108N	20 (22.2)	2	0	18 (25.4)
dhps (*n* = 90)				
Wild type	10 (11.1)	1	0	9 (12.7)
Double 437G/540E	22 (24.4)	1	0	21 (29.6)
Triple 437G/540E/581G	58 (64.4)	7	10 (100)	41 (57.7)
Combined *dhfr* and *dhps* (*n* = 90)				
Triple 51I/59R/108N+Wild type	10 (11.1)	1	0	9 (12.7)
Quadruple 51I/108N+437G/540E	12 (13.3)	0	0	12 (16.9)
Quintuple 51I/59R/108N+437G/540E	10 (11.1)	1	0	9 (12.7)
Quintuple 51I/108N+437G/540E/581G	58 (64.4)	7	10 (100)	41 (57.7)

*Withdrawals/lost to follow-up.
†Artesunate + sulphadoxine/pyrimethamine.
‡Treatment failure.
§Adequate clinical and parasitological response.

As all patients cleared their parasitemia by day 3, the presence of artemisinin resistance is unlikely. Drug selection pressure is a key prerequisite for the development of parasite resistance. SP has been available in the private sector (unpublished observation) for more than two decades. Its use as a monotherapy for the treatment of malaria likely added to the drug pressure in the parasite population. Pharmacokinetics of the drug can play an important role in the treatment outcomes in patients. Blood concentrations of the drug were not determined in the current study, and, therefore, the possibility remains that patients who experienced parasite recurrence had lower blood drug levels. Lower pyrimethamine and sulphadoxine blood levels in under-fives compared to older children and adults have been reported [14]. However, this is unlikely in this study as all patients with AS + SP treatment failure in Bosaso were aged 9 years and above.

The high prevalence of *dhfr/dhps* quintuple mutations detected in Bosaso, together with the high rates of quadruple/quintuple mutations previously found in two study sites in the southern zone in 2011 [6], indicates widespread resistance to SP in Somalia. It is interesting to note that in the current study, there was a high prevalence of the *dhps* 581G mutation occurring in combination with the *dhps* double (437G and 540E) mutation. A similarly high prevalence of this *dhps* triple mutation has been recently reported in several African countries [15–20] and is associated with SP treatment failure [17, 21]. It has been suggested that the *dhps* 581G mutation normally occurs after the *dhfr* triple (51I/59R/108N) mutation [22]. This was not true for the findings of the current study, where none of the *dhps* 581G mutations occurred with the *dhfr* triple mutation, but rather with the *dhfr* double (51I/108N). It is likely that the 581G mutation emerged in the background of *dhps* double (437G/540E), as has been suggested previously [16, 23].

Currently, nine countries recommend AS + SP as their first-line treatment (Afghanistan, Djibouti, India, Islamic Republic of Iran, Pakistan, Saudi Arabia, Somalia, Sudan and Yemen). Recent evidence has shown that AS + SP treatment was failing in north-east India, leading to its subsequent replacement [23], while high cure rates (> 95% ACPR) of the combination have been reported in Yemen [24] and Afghanistan [25].

Our findings show that AL is highly efficacious for treating uncomplicated *P. falciparum* malaria in the three study areas. Recent studies from malaria-endemic countries in Africa indicate that AL remained effective (96–100% cure rate) after many years of use [26–33].

The high AS + SP treatment failure rates, combined with the high prevalence of *dhfr-dhps* quintuple and quadruple mutations reported in the current and previous studies, called for the replacement of AS + SP with an effective ACT. In February 2016, a consensus meeting was held in Hargeisa, Somalia, where both the findings from the previous [6] and the current studies on the first-

and second-line ACTs (AS + SP and AL, respectively) were reviewed to inform treatment policy. The meeting was attended by representatives from the ministries of health from the three zones (Puntland, Somaliland and Central and South), private clinicians, local non-governmental organisations, academia, WHO and UNICEF. The meeting participants unanimously agreed to replace the first-line treatment for uncomplicated malaria (AS + SP) with AL, and to replace the second-line treatment (AL) with DHA + PPQ.

Malaria treatment with ACTs has contributed to the current decline of malaria burden [34]. Unfortunately, recent reports of artemisinin resistance in the Greater Mekong Subregion [35–37] threaten these gains, where it has been found that artemisinin resistance has both spread [35] and emerged independently [37]. With the risk of its spread or spontaneous emergence in other parts of the world, all malaria-endemic countries are urged to monitor antimalarial drug efficacy, and to be vigilant of an increase in treatment failures following treatment with an ACT, and/or an increase in the proportion of patients positive for parasitemia on day 3.

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