Efficacy of Artemether-Lumefantrine for the Treatment of *Plasmodium falciparum* Malaria in Bohicon and Kandi, Republic of Benin, 2018–2019

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Abstract. In 2005, artemether-lumefantrine (AL), an artemisinin-based combination therapy, was introduced as the first-line treatment of uncomplicated *Plasmodium falciparum* malaria in Benin. Per World Health Organization recommendations to monitor the efficacy of antimalarial treatment, we conducted a therapeutic efficacy study with AL for uncomplicated *P. falciparum* malaria in Bohicon and Kandi, Benin, from 2018 to 2019. Febrile patients aged 6 to 59 months with confirmed *P. falciparum* mono-infection received supervised doses of AL for 3 days. We monitored patients clinically and parasitologically on days 1, 2, 3, 7, 14, 21, and 28. A molecular analysis to detect mutations in the *P. falciparum* Kelch propeller gene (*Pfk13*) was carried out on day 0 samples. A total of 205 patients were included in the study. In Bohicon, the uncorrected adequate clinical and parasitological response (ACPR) proportion was 91.3% (95% confidence interval [CI]: 84.6–95.8%), whereas in Kandi this proportion was 96.7% (95% CI: 90.6–99.3%). Genotype-corrected ACPR proportions were 96.3% (95% CI: 90.9–99.0%) and 96.7% (95% CI: 90.6–99.3%) in Bohicon and Kandi, respectively. On day 3, 100% of patients in Bohicon and 98.9% of patients in Kandi had undetectable parasitemia. The C580Y mutation in the *Pfk13* gene was not observed. AL remains effective for *P. falciparum* malaria in these two sites in Benin. Monitoring antimalarial efficacy and prevalence of molecular-resistance markers in Benin should be continued to allow for early detection of antimalarial resistance and to guide treatment policies.

INTRODUCTION

Malaria is a major public health problem in Africa and other tropical areas. Approximately 229 million malaria cases and 409,000 related deaths, mostly in children under 5 years of age, occurred worldwide in 2019.¹ Benin registered approximately 2.2 million malaria cases and 2,251 deaths in 2018, mostly in children under 5 years of age.² Early diagnosis and prompt treatment with effective antimalarials are among the most important strategies for the control and prevention of malaria. Artemisinin-based combination therapy (ACT) is the standard of care to treat *Plasmodium falciparum* malaria across Africa.^{3,4} Drug resistance can adversely affect efforts to control malaria and lead to increased malaria-related mortality and morbidity. The WHO recommends monitoring of antimalarial efficacy using therapeutic efficacy studies (TESs) every 2 years in areas of high transmission to guide antimalarial treatment policies.⁵ Day 3 parasite clearance and monitoring for molecular markers of antimalarial resistance may be added to TESs as complementary components.⁶

Following international recommendations, Benin introduced artemether-lumefantrine (AL), an ACT, for treatment of uncomplicated *P. falciparum* malaria in 2005 as a result of increasing failure with chloroquine and sulphadoxine-pyrimethamine.⁷ The Ministry of Health, through the National Malaria Control Program (NMCP), has been routinely conducting TESs at several sentinel sites located in different parts of the country to monitor treatment response to the first-line

treatment, with very high observed polymerase chain reaction (PCR)-corrected efficacy findings in different parts of the country.^{8,9} However, efficacy of AL in neighboring countries has been more variable in recent years, with evidence of inadequate efficacy found in some studies.^{10–12} To guide rational antimalarial policy in Benin, a 28-day TES of AL for the treatment of uncomplicated *P. falciparum* malaria was conducted in Bohicon and Kandi. In addition, resistance marker testing was performed to detect mutations in the *P. falciparum* Kelch propeller gene (*Pfk13*) associated with artemisinin resistance.⁶

METHODS

Study timing and location. This single-arm prospective study was conducted during the malaria transmission seasons in 2018 and 2019 at Bohicon and Kandi health centers in Zou and Alibori departments, respectively (Figure 1). An assessment of laboratory records and national data revealed that approximately 424 and 305 patients are diagnosed and treated for malaria per month in Bohicon and Kandi, respectively.² Malaria patients diagnosed at these health facilities who met inclusion criteria were offered participation and were enrolled after a parent or guardian signed the study informed consent form. All parents received a copy of this form for their records.

Sample size. Each site was considered an independent sampling unit. The minimum sample size needed per site to detect a 50% treatment failure proportion with a precision of 10% and a confidence interval (CI) of 95% was 96. To account for loss to follow-up in the 28-day period, the final sample size was increased by 20%, for a target of 115 patients per site.

Study procedures. Two teams, one per site, were assembled. Each team comprised one doctor and three nurses who were responsible for screening children with malaria, two microscopists who prepared and read thin and thick smears

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FIGURE 1. Bohicon and Kandi municipalities, sites for antimalarial therapeutic efficacy monitoring, Benin, 2018–2019. This figure appears in color at www.ajtmh.org.

and other laboratory tests, and a technician to follow up with participants who did not attend scheduled study appointments. Each study team was supervised by a health zone coordinator.

The protocol for this in vivo study was based on the standard WHO methodology for surveillance of antimalarial drug efficacy.⁵ Febrile patients with a malaria blood smear positive for *P. falciparum* mono-infection at the aforementioned study sites were approached by the study team and offered participation. Study inclusion criteria included age ≤ 5 years, body weight ≥ 5 kg, hemoglobin level ≥ 5 g/dL, documented fever (axillary temperature $\geq 37.5^{\circ}\text{C}$) or recent history of fever, ability to swallow oral medication, parasite density between 2,000 and 200,000 asexual parasites/ μL , possibility and willingness of parents or guardians to follow the study protocol, and willingness to return to clinic or accept home visits during the 28-day follow-up. Exclusion criteria included presence of malaria danger signs or signs of severe malaria; history of chronic or severe underlying disease, including malnutrition; allergy to AL; and breastfeeding if mothers were receiving an antimalarial. After obtaining informed consent, demographic and clinical information was collected using standard clinical report forms (CRFs). Patients were seen on day 0 (enrollment), daily from days 1 through 3, day 7, and then weekly thereafter until day 28. Patients were also asked to return to clinic if they were ill.

At enrollment, day 0, three pairs of thick and thin smears were obtained by the study microscopist. One was stained with 10% Giemsa for fast staining and ascertainment of inclusion criteria. All other smears, including those collected on subsequent visits, were stained with 3% Giemsa for accurate parasite identification and density estimation. In addition, blood was collected by finger prick for hemoglobin determination (FaStep HB[®], AMS Laboratories, Galgorn, United Kingdom) and for preparation of dried blood spots (DBSs) on filter paper (Whatman 903 Protein Saver Cards[®], Forest Farm Industrial Estate, Cardiff, United Kingdom) for molecular tests.

The AL (20 mg artemether/120 mg lumefantrine) used in the study (Lumiter[®], Macleods Pharmaceuticals, India) was obtained by the Benin NMCP, which procured the drug after a precertification process. Treatment with AL was administered with a teaspoon of oil and followed manufacturer's weight-based dosing (5–14 kg: 1 tablet per dose, 15–24 kg: 2 tablets, 25–34 kg: 3 tablets) twice a day for 3 days. Participants received all treatment doses under direct observation and were observed for 30 minutes after each dose to monitor for vomiting or other adverse events. Those who vomited were given a second full dose and observed for additional 30 minutes. Inability to tolerate the repeat dose resulted in removal from the study and admission to the hospital for parenteral treatment.

Study teams saw participants for clinical and parasitological evaluation and the administration of the corresponding doses of AL on days 1 and 2. The day 3 visit was conducted as close to 72 hours from the first dose as possible to accurately assess for delayed parasite clearance. At each follow-up visit, the team administered a standard symptom questionnaire and conducted a physical exam, including measurement of axillary temperature, and blood was collected by finger prick for thick and thin smears, except on day 1 when no smear was collected. In case of parasitemia on day 7 or later, blood samples were collected on filter paper for molecular testing.

Hemoglobin levels were also measured on days 14 and 28 using the same method as for day 0.

Microscopic blood examination. Before study initiation, two study microscopists per site were trained for 4 days on smear preparation, staining, and microscopy reading and reporting by Benin and National Public Health Laboratory expert microscopists. During monthly supervisory visits, staff from the national laboratory read approximately 10% of slides for quality control. Parasite density was estimated by counting the number of asexual parasites against 200 white blood cells (WBCs) in the thick smear and estimated in parasites/ μL based on an estimated WBC count of 6,000 WBC/ μL . When fewer than 100 parasites counted at the 200 WBCs mark, counting continued until 500 WBCs were seen. A total of 1,000 WBCs were counted before a blood smear was considered negative. Smears were read by two independent microscopists blinded to each other; in case of discordance in species determination or if asexual parasite density estimates differed by more than 25%, a third microscopist read the slide. Asexual parasite density was reported as a geometric mean of the two concordant readers. Gametocyte presence was reported as detectable or undetectable.

Study outcomes. The WHO definition of therapeutic responses was used to classify participant outcomes.⁵ In summary, early treatment failure (ETF) was defined as having signs of severe malaria on days 1–3, asexual parasite density on day 2 higher than on day 0, parasitemia on day 3 with axillary temperature $\geq 37.5^{\circ}\text{C}$, or parasitemia on day 3 $\geq 25\%$ of day 0. Late treatment failure was classified as either late clinical failure (LCF) or late parasitological failure (LPF). LCF was defined as parasitemia in the presence of either danger signs of severe malaria or axillary temperature $\geq 37.5^{\circ}\text{C}$ on any day between day 4 and 28 in participants who did not previously meet criteria for ETF. LPF was defined as presence of parasitemia on any day between day 7 and 28 in the absence of fever or any criteria for ETF or LCF. Adequate clinical and parasitological response (ACPR) was defined as absence of parasitemia on day 28 in participants who did not previously meet any of the criteria for ETF, LCF, or LPF. Participants who met criteria for ETF, LCF, or LPF were removed from the study and treated with a rescue regimen (parenteral artesunate, artemether, or quinine) per Benin treatment guidelines.⁷

Molecular testing. To distinguish recrudescences from new infections, genotyping was conducted at the Molecular Biology and Genomics Laboratory at Aristide Hospital DAN-TEC in Dakar, Senegal. Genomic DNA was extracted from filter paper punches using the manufacturer's protocol for Qiagen DNA Blood kit (Qiagen, Hilden, Germany). First, we performed molecular diagnostic confirmation using nested PCR for day 0 and day of recurrence samples to confirm *P. falciparum* infection.^{13,14} Then, to differentiate recurrences into reinfections and recrudescences, genotyping was performed using primers to the central block of the merozoite surface proteins 2 (*mSP2*), the glutamate rich protein (*glurp*), and the block 2 of the merozoite surface proteins 1 (*mSP1*) genes, in that order.¹⁵ Amplification products were separately on a 2% agarose gel impregnated with ethidium bromide and visualized under ultraviolet light. If paired samples did not have at least one match, that is, a band with the exact same size upon gel examination, for a given gene, the subsequent amplifications were not conducted, and the paired samples were considered heterologous and associated recurrence deemed reinfection.

in the genotype-corrected analysis. Recurrences for which paired samples shared at least one size-matched band for each one of the three genes were considered recrudescences in the genotype-corrected analysis.

We used the high-resolution melting (HRM) technique to genotype the C580Y mutation of the *Pfk13* gene of all day 0 samples.¹⁶ Two DNA reference samples (NF54 transgenic line with 580Y mutant allele and the clone 7 wild-type C580) were used to detect the polymorphism. Study samples were diluted to 10 ng/ μ L in standard grade water. We added working stock solution with forward and reverse primers and probes, HRM Light Scanner[®] Master Mix (Idaho Technology Inc., Salt Lake City, UT), and PCR-grade water to DNA template for a total reaction volume of 10 μ L. The reaction was run on the Light Cycler 96 (Roche Molecular Systems[®], Pleasanton, CA) using 55 cycles at 95°C for 2 seconds and 56°C for 15 seconds. HRM data were analyzed as described previously.¹⁶ Samples were classified as wildtype if they possessed the same nucleotide sequence as our reference strain or mutant if not.

Statistical analysis. A CRF data entry form was developed in Census and Survey Processing System[®] version 7.2 (U.S. Census Bureau, Suitland, MD) and validated by the Laboratory and Chemosensitivity team within the Benin NMCP. Four data entry officers were responsible for double entry and data cleaning in collaboration with the Benin NMCP. We analyzed data using SAS version 9.3 (SAS Institute, Cary, NC). Descriptive data are presented as percentages, means, standard deviation, median, and ranges, as appropriate.

The primary study endpoints were the day 28 uncorrected and genotype-corrected ACPR proportions by per-protocol analysis.⁵ For these, the denominator included only participants with ACPR or treatment failure and excluded those with loss to follow-up or protocol violation. For the genotype-corrected analysis, the numerator and denominator also excluded recurrences on or after day 4 classified as reinfections by genotyping. In addition, we calculated cumulative success rate, estimated by genotype-corrected Kaplan-Meier analysis, considering all participants until they were censored, that is, recrudescence, reinfections, withdrawal, loss of follow-up, and ACPR.

Ethical considerations. The study was reviewed and approved by the National Health Research and Ethics Committee before starting of the study (Protocol BEN/004/2018/NMCP). The Associate Director for Science, Center for Global Health, Centers for Disease Control and Prevention (CDC) also reviewed this proposal and considered CDC staff to be nonengaged in this research activity (Project no. 2017-141). Before inclusion, written informed consent from a participant's legal guardian was obtained. In addition, consent could be withdrawn at any time from the study without causing harm to a participant's care.

RESULTS

Enrollment. Of the 301 patients visiting either health facility from October 2018 to February 2019 who screened positive for *P. falciparum*, 145 participants were enrolled, 115 in Bohicon and 30 in Kandi. Because of the low enrollment rate in Kandi, a second patient enrollment was undertaken for that site, and an additional 60 patients were admitted after screening additional 100 patients from June to September 2019

(Figure 2). All positive samples were confirmed as *P. falciparum* by PCR.

Baseline characteristics. Participant median ages for Bohicon and Kandi were 30 and 41 months, respectively (Table 1). About half of participants were male (51.3% in Bohicon and 51.1% in Kandi). All participants had fever at the enrollment visit; mean temperature at enrollment was 38.7°C and 38.6°C for Bohicon and Kandi, respectively. These and other variables at enrollment are listed in Table 1.

Side effects and patient follow-up. Adverse events reported during AL treatment included nausea, abdominal pain, and vomiting. Only one participant vomited after having received a dose of medication; that participant tolerated the repeat dose. No participant was lost to follow-up; therefore, the uncorrected per-protocol analysis considered 115 and 90 participants in Bohicon and Kandi, respectively.

The arithmetic mean of asexual parasite densities at baseline was 46,424 (range: 611–199,603) and 59,774 (range: 2,100–199,010) in Bohicon and Kandi, respectively (Table 2). The proportion of participants with asexual parasitemia declined from 11.3% to 0% from day 2 to 3, respectively, in Bohicon, with similar decreases in Kandi (3.4% to 1.1%, respectively). Data on mean parasite densities during follow-up are listed in Table 2. Mean hemoglobin levels increased from 7.9 g/dL on day 0 to 8.5 g/dL and 9.4 g/dL on days 14 and 28, respectively, in Bohicon. In Kandi, hemoglobin levels were 8.6 g/dL, 9.0 g/dL, and 10.2 g/dL on days 0, 14, and 28, respectively. This increase in both sites was statistically significant ($P < 0.001$).

Therapeutic efficacy. In Bohicon, 10 of 115 patients had recurrences classified as treatment failures in the uncorrected analysis; six patients had LPF and four LCF. The uncorrected ACPR was 91.3% (95% CI: 84.6–95.8%) in the per-protocol analysis and 91.3% (95% CI: 85.5–95.7%) in the Kaplan-Meier analysis. On the basis of genotyping analysis, four of these were classified as recrudescences, and six were classified as reinfections. Thus, the genotype-corrected ACPR considered four failures and 109 patients and was estimated at 96.3% (95% CI: 90.9–99.0%), and the corresponding Kaplan-Meier estimate was 96.3% (95% CI: 92.0–99.0%).

In the uncorrected analysis, three of 90 patients had treatment failures in Kandi: one with ETF and two with LCF. Uncorrected ACPR was 96.7% (95% CI: 90.6–99.3%) in the per-protocol analysis and 96.7% (95% CI: 92.0–99.4%) in the Kaplan-Meier analysis. The two patients with LCF in the uncorrected analysis were found to be of the same genotype of those of day 0 and were classified as recrudescences. Therefore, we did not exclude any patient for the genotype-corrected ACPR analysis, reaching a per-protocol ACPR of 96.7% (95% CI: 90.6–99.3%) and a corresponding Kaplan-Meier ACPR of 96.7% (95% CI: 92.0–99.4%). Raw genotyping data are available in supplemental materials. Figure 3 shows the genotype-corrected survival curve for both study sites using day 28 ACPR as the outcome of interest.

Molecular markers of resistance. No C580Y mutation in the *PfK13* gene was observed because all genotyped isolates had the wildtype C580 allele.

DISCUSSION

We found that AL remains highly efficacious for treatment of uncomplicated *P. falciparum* malaria in two sites in Benin after

FIGURE 2. Screening, enrollment, and follow-up of study participants, Bohicon and Kandi, Benin, 2018–2019 ($N = 205$). Note: In Kandi, 90 patients screened in the first enrollment phase in 2018 and 100 in the second in 2019 (see text for details).

16 years of AL use in Benin, with day 28 genotype-corrected ACPR of 96.3% and 96.7% in Bohicon and Kandi, respectively, in the per-protocol analysis. Day 3 parasitemia was not observed in Bohicon and was observed in only one (1.1%) participant in Kandi, a low frequency that is consistent with good efficacy of the artemisinin component in this combination. Our study represents an effort by the Benin NMCP and its partners to monitor antimalarial efficacy in the country.

Our results are reassuring because the WHO-recommended threshold to consider a change in national antimalarial policy is an ACPR < 90%.^{5,17} A prior study in Benin conducted in 2014 showed molecular-correct ACPR of 100% among children under 5 years of age treated with AL.¹⁸ The high AL efficacy might be related to the efforts to support the judicious use of antimalarials in Benin, with adherence to malaria testing results and to the combination therapy regimen itself, so that resistance to its components does not develop as it would if they were used as monotherapy or for insufficient number of days. There have been recent studies in neighboring Nigeria and Ghana with similarly high AL efficacy.^{11,19} However, other results from Ghana and Burkina Faso in West Africa and Angola in southern Africa have observed reduced efficacy of AL in some regions, although those findings seem to be restricted to certain provinces.^{12,19–21} This variability in efficacy, with results close to the WHO threshold for treatment policy revision in some instances, speaks to the focal nature of antimalarial resistance

and the importance of monitoring antimalarial efficacy across different areas in a country.

We noted a low hemoglobin level among our patients at enrollment, but that improved during follow-up. This finding is in line with studies in nearby Ghana where, after malaria treatment, hemoglobin levels improved rapidly.^{10,19} Prior studies in Benin have showed persistence of low levels of hemoglobin after malaria treatment. Our findings might signal recent improvements in child health programs addressing other causes of anemia in children, such as geohelminths.¹⁸ It also shows that malaria seems to be a main contributor to anemia in children and underscores the need for combating malaria as a means to improve childhood health markers.

Our study has a few limitations. We did not systematically collect information about adverse events associated with AL use, so we cannot accurately ascertain the frequency of these events. However, considering that only one patient in Kandi and none in Bohicon vomited after a treatment dose, we cautiously assume treatment tolerance was high, as in other studies in the region.²² We only evaluated the presence of the C580Y *Pfk13* gene mutation. Other K13 mutations are now being observed at higher frequencies in some countries in Africa. Sanger sequencing in addition to other techniques for detection of *Pfk13* gene mutations, such as amplicon deep sequencing, would have allowed for detection of other mutations in the gene associated with artemisinin resistance.²³ We also did not evaluate other molecular markers, such as

TABLE 1
Characteristics of study participants at enrollment, Bohicon and Kandi, Benin, 2018–2019 ($N = 205$)

Variable	Value	
	Bohicon	Kandi
Patients enrolled, n	115	90
Age, months, median (range)	30 (6–58)	41 (6–59)
Male, n (%)	59 (51.3)	46 (51.1)
Weight (kg), mean (SD)	12.3 (2.8)	14.0 (3.3)
Body temperature, °C, mean (SD)	38.7 (0.9)	38.6 (0.8)
Hemoglobin (g/dL), mean (SD)	7.9 (2.0)	8.6 (1.9)
Asexual parasite density on day 0, parasites/ μ L, arithmetic mean (range)	46,424 (611–199,603)	59,774 (2,100–199,010)
Gametocytes presence on day 0, n (%)	1 (0.9)	0 (0)

TABLE 2

Asexual parasite presence and density, and gametocyte presence by day of follow-up, Bohicon and Kandi, Benin, 2018–2019 (N = 205)

Day	Bohicon			Kandi		
	Asexual parasite presence and parasite density*		Gametocyte presence	Asexual parasite presence and parasite density*		Gametocyte presence
	Positive, n (%)	Parasites/μL, arithmetic mean (range)		Positive, n (%)	Parasites/μL, arithmetic mean (range)	
0	115 (100%)	46,424 (611–199,603)	1 (0.9)	90 (100%)	59,774. (2,100–199,010)	0 (0%)
2	13 (11.3%)	41.2 (23–104)	7 (6.1%)	3 (3.4%)	11,901.0 (431–24,512)	0 (0%)
3	0 (0%)	0	0 (0%)	1 (1.1%)	82 (82–82)	0 (0%)
7	0 (0%)	0	0 (0%)	0 (0%)	0	0 (0%)
14	0 (0%)	0	0 (0%)	0 (0%)	0	0 (0%)
21	6 (5.21%)	57,201.2 (5,804–23,025)	1 (0.9%)	2 (2.22%)	246,512.5 (233,025–260,000)	0 (0%)
28	2 (1.7%)	36,975.0 (2,830–71,436)	2 (1.7%)	0 (0%)	0	0 (0%)

*Parasite means include microscopy-positive samples only.

P. falciparum multidrug-resistant 1 (*pfmdr1*) gene, associated with decreases in lumefantrine susceptibility in some studies, which would have allowed us to also infer presence of resistant markers to the partner drug of the ACT used in Benin.^{24,25} Considering seasonal chemoprevention with sulfadoxine-pyrimethamine and amodiaquine is used in parts of Benin, resistance markers to these drugs should also be considered for samples collected as part of in vivo studies. However, the lack of the genetic information does not hamper our conclusions because genotype corrected ACPR remains the main outcome for TESs.

We recognize TESs are the gold-standard methodology for antimalarial efficacy monitoring and policy decision-making.⁵ Conducting TESs at the required intervals—every 2 years—is not a trivial task for malaria control programs because it requires allocating resources, personnel, and commitment from multiple partners, especially for required genetic testing. Benin NMCP has been able to conduct four rounds of TESs from 2007 to 2018; results from two have been publicly disseminated. The last two rounds of TESs also included genotyping correction. Obstacles to timely TES data analysis and reporting should be evaluated and addressed so malaria

control programs can consider changes to their treatment policies as early as warranted.

We recognize the importance of collecting antimalarial efficacy from different regions of a country, as efficacy can vary and pockets with distinct resistance profiles may be present.^{19,26} As countries move forward to regular and sustainable collection of antimalarial efficacy and resistance information, it is important to find the right balance on selecting sites for longitudinal monitoring and also improve in-country capacity to implement and supervise TES and perform molecular tests for genotyping and resistance markers. This will allow for early detection of antimalarial resistance and support evidence-based antimalarial policies.

In summary, our study found evidence of continued high efficacy of AL in Benin, which supports its ongoing use as first-line therapy for uncomplicated *P. falciparum* malaria in the country. However, regular in vivo monitoring and molecular surveillance of mutations in genes associated with artemisinin (*Pfk13*) and partner drug resistance in the region are recommended every 2 years per WHO guidelines to inform antimalarial treatment policies.

Received January 22, 2021. Accepted for publication April 19, 2021.

Published online July 12, 2021.

Note: Supplemental material appears at www.ajtmh.org.

Acknowledgments: We thank the participants and their caregivers who kindly agreed to the 28-day follow-up; study personnel who made it possible to enroll and follow patients; and the staff at Bohicon and Kandi health centers for their collaboration and support. We are also indebted to all the team of the Molecular Biology and Genomics Laboratory at Aristide Hospital DANTEC in Dakar, Senegal, for their welcome and their technical assistance and supervision of the molecular biology component, specially Mouhamad Sy, Tolla Ndiaye, and Amy Gaye. Thank you to Dr. Alexandre Macedo for his technical review of the manuscript. Finally, many thanks to Dr. Cyriaque Degbe and Dr. Razac Osse for their technical review of the study protocol as part of the ethical approval process in Benin.

Financial support: Funding for this study was partially provided by the U.S. Agency for International Development through the President's Malaria Initiative.

Disclaimer: The funding sources for this study had no role in study design, data collection, analysis, or interpretation. The opinions expressed herein are those of the authors and do not necessarily reflect the views of the Benin National Malaria Control Program, U.S. Agency for International Development, or the Centers for Disease Control and Prevention.

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FIGURE 3. Genotype-corrected day 28 adequate clinical and parasitological response (ACPR) survival curve per site, Bohicon and Kandi, Benin (N = 205). This figure appears in color at www.ajtmh.org.

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