MAJOR ARTICLE

Impact of Daily Cotrimoxazole on Clinical Malaria and Asymptomatic Parasitemias in HIV-Exposed, Uninfected Infants

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Background. Cotrimoxazole preventive therapy (CPT) is recommended for all human immunodeficiency virus (HIV)–exposed infants to avoid opportunistic infections. Cotrimoxazole has antimalarial effects and appears to reduce clinical malaria infections, but the impact on asymptomatic malaria infections is unknown.

Methods. We conducted an observational cohort study using data and dried blood spots (DBSs) from the Breastfeeding, Antiretrovirals and Nutrition study to evaluate the impact of CPT on malaria infection during peak malaria season in Lilongwe, Malawi. We compared malaria incidence 1 year before and after CPT implementation (292 and 682 CPT-unexposed and CPT-exposed infants, respectively), including only infants who remained HIV negative by 36 weeks of age. Malaria was defined as clinical, asymptomatic (using DBSs at 12, 24, and 36 weeks), or a composite outcome of clinical or asymptomatic. Linear and binomial regression with generalized estimating equations were used to estimate the association between CPT and malaria. Differences in characteristics of parasitemias and drug resistance polymorphisms by CPT status were also assessed in the asymptomatic infections.

Results. CPT was associated with a 70% (95% confidence interval, 53%–81%) relative reduction in the risk of asymptomatic infection between 6 and 36 weeks of age. CPT appeared to provide temporary protection against clinical malaria and more sustained protection against asymptomatic infections, with no difference in parasitemia characteristics.

Conclusions. CPT appears to reduce overall malaria infections, with more prolonged impacts on asymptomatic infections. Asymptomatic infections are potentially important reservoirs for malaria transmission. Therefore, CPT prophylaxis may have important individual and public health benefits.

Keywords. antifolate resistance; cotrimoxazole; HIV; infant; malaria.

The World Health Organization (WHO) recommends that all human immunodeficiency virus (HIV)–exposed infants begin cotrimoxazole preventive therapy (CPT) between 4 and 6 weeks of age, and continue CPT until at least 6 weeks after cessation of breastfeeding and until HIV infection has been ruled out [1]. CPT is used to avoid often-fatal opportunistic infections among children born to HIV-infected women, and in turn reduces hospital admissions. CPT use among HIV-infected adults and children has been associated with reductions in malaria incidence, as cotrimoxazole has antimalarial activity [2–4]. Similarly, CPT has been associated with reduced incidence of clinical malaria among HIV-exposed but uninfected (HEU) infants, with some suggestion that protective benefits may wane over time [5–7]. As early infant HIV diagnosis services are becoming more widely available in Africa, and as more efficacious drug regimens for the prevention of mother-to-child HIV transmission are being adopted

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and extended throughout the breastfeeding period, the population of HEU infants is growing. A better understanding of the impact of CPT on malaria among HEU infants is needed.

To date, only clinical or symptomatic malaria has been examined in studies of CPT use and malaria incidence. However, clinical malaria represents only a portion of all malaria infections, as many malaria infections are asymptomatic. Although asymptomatic infections are more common in older children, they do occur in infants. We assessed the association between CPT and a composite outcome of clinical or asymptomatic infection. Additionally, we evaluated the association between CPT and both clinical and asymptomatic infections separately. Including asymptomatic infection adds valuable information concerning the routine use of CPT among the growing population of HEU infants, as the role of asymptomatic parasitemia in malaria transmission has garnered increasing attention. Although density of parasitemia and gametocytemia affects transmission efficiency [8], transmission may occur from asymptomatic patients and patients with submicroscopic parasitemia [9].

In addition to assessing the impact of CPT on malaria infection, we also compared characteristics of the asymptomatic infections by CPT status. Specifically, we assessed (1) parasitemia levels; (2) complexity of individual infections, as in high-transmission areas such as Malawi infections are often polyclonal; and (3) frequency of single-nucleotide polymorphisms in *dhfr* and *dhps*, the genes associated with resistance to antifolate antimalarials. Because resistance to antifolate antimalarials is common in Africa, understanding how CPT affects parasite characteristics and works in the presence of high levels of antifolate resistance is important.

METHODS

Study Design

We used data and infant dried blood spot (DBS) specimens from the Breastfeeding, Antiretrovirals, and Nutrition (BAN) study, which has been described elsewhere [10, 11]. Beginning in June 2006, 240 mg of cotrimoxazole was provided once daily to all HIV-exposed infants 6 weeks after birth, or as soon as possible thereafter, and continued through 36 weeks of age due to compliance with Malawian national guidelines and WHO recommendations. Infants reaching 36 weeks of age prior to June 2006 did not routinely receive CPT. To compare malaria incidence 1 year before and after implementation of CPT, we included HEU infants born between 22 August 2004 and 21 August 2005 (CPT unexposed, n = 292), and those born between 22 August 2006 and 21 August 2007 (CPT exposed, n = 682). Dates were chosen to ensure that all infants were at least 6 weeks of age (and therefore eligible for CPT in the later time period) by the start of peak malaria season (October), and to mitigate any confounding effects of calendar time. Infant HIV-1 status was determined by Roche Amplicor 1.5 DNA polymerase chain reaction (PCR) (Roche Molecular Systems, Pleasanton, California) at 2, 12, 28, and 48 weeks. PCR-positive results were confirmed by testing an additional blood specimen, and the window of infection was narrowed with tests of infant DBS specimens taken at 4, 6, 8, 18, 24, 32, and 36 weeks. All mothers were advised to breastfeed exclusively for the first 24 weeks postpartum, and to wean between 24 and 28 weeks.

Only malaria events occurring during peak malaria season (October–April) were used to increase the likelihood of detecting clinical and asymptomatic parasitemias. Infants born between 22 August 2005 and 21 August 2006 were excluded to avoid exposure misclassification while the new CPT policy was being implemented. Infants who were diagnosed as HIV infected between birth and 36 weeks of age (n = 84) or had a documented episode of clinical malaria before 6 weeks of age (n = 8) were excluded. A total of 882 infants of the 2369 mother–infant pairs enrolled in the BAN study cohort met eligibility criteria.

Data Analyses

Malaria was defined 3 ways: (1) clinical, (2) asymptomatic, and (3) a composite outcome of clinical and asymptomatic. Clinical malaria was defined as having clinical symptoms of malaria with documented microscopy-positive parasitemia between 6 and 36 weeks of age (n = 148). In addition, infants with a documented serious adverse event due to malaria were considered as having clinical malaria even if documented microscopy-positive parasitemia was unavailable (n = 4). To assess for changes in the association between CPT and malaria by infant age, clinical malaria was also categorized as occurring at 6–12, 13–24, or 25–36 weeks of age. If an infant had multiple malaria events, only events occurring >1 week apart were counted as a recurrent event and used in analyses.

Asymptomatic infection was determined from available DBS specimens collected during peak malaria season. A total of 471, 517, and 564 infants were 12, 24, and 36 weeks of age, respectively, during peak malaria season. Among these, DBSs were available for 230 (49%) infants at 12 weeks of age, 235 (45%) at 24 weeks, and 215 (38%) at 36 weeks. Infants who were missing DBSs at 12, 24, or 36 weeks did not differ from infants who had an available DBS, with respect to characteristics outlined in Table 1. Missing asymptomatic infection information was therefore considered missing completely at random. Samples testing positive for malaria parasites by 2 previously described real-time PCR assays were considered positive [12]. Samples positive with only 1 assay were considered indeterminate and not used in analyses. Asymptomatic infection was treated as a dichotomous variable (detectable vs undetectable parasitemia).

Table 1. Baseline Characteristics of Mother–Infant Pairs

Characteristic	Total (N = 882)	No Malaria[a] (n = 701)	Malaria[a] (n = 181)
Antiretroviral randomization			
Maternal antiretroviral	294 (33)	235 (34)	59 (33)
Infant nevirapine	297 (34)	239 (34)	58 (32)
Enhanced control	291 (33)	227 (32)	64 (35)
Nutritional randomization			
No supplement	449 (51)	357 (51)	92 (51)
Received supplement	433 (49)	344 (49)	89 (49)
Mothers[b]			
Age, y			
15–25	436 (50)	355 (51)	81 (45)
26–35	404 (46)	312 (45)	92 (51)
36–45	39 (4)	33 (5)	6 (3)
Parity			
0	133 (15)	109 (16)	24 (13)
≥1	748 (85)	591 (84)	57 (87)
Education			
Primary school only	567 (64)	441 (63)	126 (70)
More than primary school	314 (36)	260 (37)	54 (30)
Infants[b]			
Sex			
Female	443 (50)	350 (50)	93 (51)
Male	439 (50)	351 (50)	88 (49)
Birth weight, kg			
<2.5	66 (8)	56 (8)	10 (6)
≥2.5	814 (93)	643 (92)	171 (94)
Hemoglobin, g/dL			
<13.4	54 (6)	44 (6)	10 (6)
≥13.4	828 (94)	657 (94)	171 (94)
Neutropenia, $\times 10^3$ cells/μL			
<1	23 (3)	18 (3)	5 (3)
≥1	859 (97)	683 (97)	176 (97)

Data are presented as No. (%).

[a] Malaria defined as clinical or polymerase chain reaction–positive malaria.

[b] Maternal age missing for 3 mother–infant pairs, parity and maternal education missing for 1 mother–infant pair, infant birthweight missing for 2 infants. Percentages may not add to 100 due to rounding.

We compared maternal malaria prevalence by time period to assess for any confounding effects of malaria transmission differences across calendar time. Malaria exposure can vary greatly across small geographic distances [13, 14]. Maternal malaria prevalence was therefore thought to provide a more valid proxy of transmission differences over time, compared to malaria prevalence data for the community at large. Maternal malaria prevalence was calculated using clinical malaria data among mothers with a CD4 count >500 cells/μL and therefore not receiving routine maternal CPT.

Frequencies, means, and medians were calculated, as appropriate, to compare characteristics of mother–infant pairs by exposure (CPT status) and outcome (malaria) category. Binomial regression models using generalized estimating equation (GEE) with an autoregressive correlation structure were used to estimate risk ratios (RRs) for malaria by CPT status when malaria was treated as a dichotomous variable. Linear regression with GEE was used when asymptomatic infection was treated as a continuous variable. Mann–Whitney test was used to assess differences in median parasitemia level and median multiplicity of infection (MOI) by CPT status, Fisher exact test was used to assess differences in maternal malaria prevalence by time period, and unpaired *t* test was used to assess differences in presence of resistance polymorphisms.

The following parameters were assessed for potential confounding or effect measure modification: BAN study antiretroviral and nutritional randomization assignment, maternal characteristics, infant health status information, and maternal malaria prevalence. Parameters were decided based on expert knowledge and use of a causal diagram to identify a minimally sufficient adjustment set [15]. Effect measure modification was assessed by comparing unadjusted and adjusted estimates and 95% confidence intervals (CIs) using an interaction term between CPT and the variable of interest. Covariates that produced adjusted estimates that were different enough to be clinically or programmatically relevant were considered effect measure modifiers. Among variables identified in the minimally sufficient adjustment set, confounding was assessed using a change-in-estimate approach. If the change-in-estimate of the odds ratio was >10% ($|\ln(OR_{reduced}/OR_{full})| > 0.10$), and the covariate was not an effect measure modifier, the covariate was treated as a confounder. All data analyses were conducted using SAS version 9.3 (SAS Institute, Cary, North Carolina).

Molecular Analysis of Asymptomatic Parasitemias

Molecular analysis was performed on all DBSs to determine asymptomatic parasitemias. We determined the parasitemia of each infection using real-time PCR to detect the single-copy gene *Plasmodium falciparum* lactate dehydrogenase (*pfldh*) as previously described [16]. Parasitemia data by microscopy for clinical events was not recorded and no biologic specimens were available to perform molecular analysis. Complexity of infection was assessed by nested PCR amplification of merozoite surface protein 2 (*msp2*) as previously described [17]. The amplified fragments were sized by capillary electrophoresis using the High Sensitivity DNA1000 kit on an Agilent Tape Station 2200 (Agilent Technologies, Santa Clara, California). Bands representing >10% of the height of the largest peak in the sample were counted. Drug resistance polymorphism frequency was determined using a modified version of a previously described pooled deep sequencing approach [18, 19]. This approach uses

pools created by mixing equal volumes of extracted DNA from each sample and has been shown to create accurate allele frequency measurements compared to Sanger sequencing of individual samples [18]. Pools for participants exposed and unexposed to CPT were created and amplified in duplicate for the *dhfr* and *dhps* regions containing key sulfadoxine-pyrimethamine resistance polymorphisms. The *dhfr* region was amplified using sequence-specific primers as previously reported [18]. The *dhps* region was amplified using 800 nM primer 437-F (TGAAATGATAAATAAGGTGCTAGTGT), 800 nM primer 613-R (GTTGTGTATTTATATTTTGATCATTC), KAPA HiFi Fidelity Buffer (KAPA Biosystems, Boston, Massachusetts), 400 μM KAPA dNTP Mix, 1 U of KAPA HiFi HotStart DNA Polymerase, and 5 μL of sample DNA with the following cycling parameters: 95°C for 3 minutes; 40 cycles of 98°C for 20 seconds, 63°C for 20 seconds, and 72°C for 60 seconds; and extension at 72°C for 2 minutes with a 4°C hold. The PCR products were then sheared as previously described [19], size selected by E-gel (Life Technologies, Grand Island, New York) and library prepped by the Ion Plus Fragment Library Kit (Life Technologies). The indexed libraries were sequenced on a 314 chip using an Ion Torrent PGM (Life Technologies). Read alignment and scoring of allele frequency were done as previously described [18]. The following polymorphic sites were evaluated: N51I, C59R, S108N, and I164L in *dhfr* and S436A, S436Y, S463F, A437G, K540E, A581G, A613S, and A613T in *dhps*.

RESULTS

CPT and Malaria

Characteristics of study participants are shown in Table 1. In addition, prevalence of clinical malaria among mothers with a CD4 count >500 cells/μL did not significantly differ by time period (23% in CPT-unexposed group, 19% in CPT-exposed group; $P = .4$).

The primary outcome was episodes of malaria, defined as a combination of clinical and/or asymptomatic infections. A total of 181 (21%) infants experienced at least 1 episode of clinical (n = 137) and/or asymptomatic (n = 61) infection, 11 infants had 2 episodes of clinical malaria, and 2 infants had 3 episodes of clinical malaria between 6 and 36 weeks of age during peak malaria season. Twelve infants experienced an episode of both asymptomatic infection and clinical malaria, with 5 infants experiencing both a clinical and asymptomatic infection event within a time interval (4 at 13–24 weeks and 1 at 25–36 weeks). In all cases, the asymptomatic event occurred >4 weeks after the clinical event and, therefore, both events were included in analyses. In total, 67 CPT-unexposed infants (26%) and 114 CPT-exposed infants (18%) experienced at least 1 episode of clinical malaria or asymptomatic infection.

Table 2. Association Between Cotrimoxazole Preventive Therapy and Both Clinical Malaria and Asymptomatic Infection, by Infant Age

Type of Infection	No. of Events	RR (95% CI)
Clinical or asymptomatic infection		
Daily infant CPT vs no infant CPT		
6–12 wk	30	0.38 (.19–.77)
13–24 wk	80	0.53 (.34–.80)
25–36 wk	98	0.96 (.63–1.48)
Overall: 6–36 wk	208	0.65 (.49–.86)
Clinical malaria only		
Daily infant CPT vs no infant CPT		
6–12 wk	18	0.33 (.13–.82)
13–24 wk	40	0.85 (.44–1.63)
25–36 wk	94	0.96 (.62–1.49)
Overall: 6–36 wk	152	0.81 (.58–1.15)
Asymptomatic infection only		
Daily infant CPT vs no infant CPT		
12 wk	12	0.26 (.09–.78)
24 wk	44	0.32 (.19–.55)
36 wk	5	0.14 (.02–1.27)
Overall: 12–36 wk	61	0.30 (.19–.47)

Abbreviations: CI, confidence interval; CPT, cotrimoxazole preventive therapy; RR, risk ratio.

Overall, daily infant CPT was associated with 0.65 (95% CI, .49–.86) times the risk of having clinical or asymptomatic infection between 6 and 36 weeks of age during peak malaria season, corresponding to a 35% (95% CI, 14%–51%) relative reduction in malaria (Table 2). When combining all time intervals and evaluating clinical malaria separately, daily CPT use was not associated with a statistically significant decrease in the relative risk of clinical malaria (RR, 0.81 [95% CI, .58–1.15]). However, CPT was associated with a large decrease in the relative risk of asymptomatic infection from 12 to 36 weeks (RR, 0.30 [95% CI, .19–.47]). Adjustment for infant birth weight and antiretroviral randomization had little to no impact.

The relative reduction in malaria risk differed by infant's age, with CPT having a stronger effect on malaria risk during the first few months of life and decreasing thereafter (Table 2). Comparing malaria type (clinical vs asymptomatic), daily CPT was associated with a 67% (95% CI, 18%–87%) relative reduction in the risk of clinical malaria between 6 and 12 weeks of infant age, but no statistically significant relative reduction thereafter. Daily CPT was associated with a 68%–86% relative reduction in the risk of asymptomatic infection during peak malaria season, compared with not receiving daily CPT. However, the relative reduction was not statistically significant at 36 weeks of age, likely due to the small number of asymptomatic infection events at 36 weeks (n = 5).

Table 3. Frequency of Antifolate Resistance Mutations in Infants Unexposed or Exposed to Cotrimoxazole Preventive Therapy

Resistance Mutation	Replicate 1	Replicate 2	Mean	SD	*P* Value[a]
dhfr N51I					
CPT unexposed	98.6	97.4	98.0	0.8	.1
CPT exposed	99.5	99.4	99.5	0.1	
dhfr C59R					
CPT unexposed	96.6	96.5	96.6	0.1	.6
CPT exposed	96.0	96.7	96.4	0.5	
dhfr S108N					
CPT unexposed	99.7	99.6	99.7	0.1	.04
CPT exposed	99.9	99.9	99.9	0.0	
No. of reads					
dhfr	35 211	22 573	28 892	8936	
dhps	17 885	20 400	19 143	1778	

Abbreviations: CPT, cotrimoxazole preventive therapy; *dhfr*, dihydrofolate reductase; *dhps*, dihydropteroate synthetase; SD, standard deviation.

[a] *P* value based on an unpaired *t* test.

Impact of CPT on the Characteristics of Asymptomatic Infection

All infections detected by molecular methods in this study were *P. falciparum* infections. We were able to determine a parasitemia from all 61 available samples (CPT unexposed: 35 samples, CPT exposed: 26 samples; geometric mean: 2.75 genome equivalents/μL). When parasitemia occurred, the parasitemia levels were similar by CPT status (mean difference, −0.20 [95% CI, −.67 to .27]). Complexity of infection was determined for 58 of 61 (95%) samples. The use of CPT was not associated with a decrease in the MOI, with a mean MOI of 1.7 in the treated infants and 1.9 in the untreated infants ($P = .5$).

In total, 224 659 sequences spanning *dhfr* and *dhps* passed the sequencing quality and mapping filters, with a mean number of sequences per PCR of 28 082 (range, 17 885–36 213). All sites evaluated for known drug polymorphisms (listed in last sentence of "Methods" section) were covered by >1000 sequencing reads. Mutant alleles were seen only at 4 loci: *dhfr* N51I, C59R, and S108N, as well as *dhps* A437G. All other sites were pure wild type. Allele frequency for mutant alleles is shown in Table 3. Of note, sulfadoxine-pyrimethamine (Fansidar) was used as first-line treatment for uncomplicated malaria from 1993 to 2007, replacing chloroquine. Artemether-lumefantrine was officially adopted as first-line treatment in 2007 [20].

DISCUSSION

During peak malaria season, daily CPT given to HEU infants was associated with an overall reduction in the risk of malaria infection between 6 and 36 weeks of life. This adds to our previous findings that CPT is associated with reduced risk of severe clinical malaria year round, and reduced nonsevere clinical malaria in the first 10 weeks of life [5, 21]. However, we now provide the first evaluation of the impact of CPT on incidence and characteristics of asymptomatic infections among HEU infants. CPT remained effective at preventing asymptomatic infection even in the setting of very high sulfadoxine-pyrimethamine resistance. Reduction in malaria incidence occurred without significant changes in parasitemia levels, the complexity of infections, or the prevalence of drug resistance mutations.

Protection from asymptomatic parasitemia is important for several reasons. First, asymptomatic infections frequently become symptomatic [22, 23]. Second, it is likely that asymptomatic infections are not truly asymptomatic. There is concern that even asymptomatic infections may have measurable adverse clinical effects, in particular, hematologic abnormalities among children and pregnant women [24–27]. Last, concerns remain regarding the impact of asymptomatic infection on transmission. Transmission may occur from asymptomatic patients and patients with submicroscopic parasitemia [28, 29]. In feeding studies aimed at demonstrating the contribution of asymptomatic infections to transmission, 1.2% of mosquitoes feeding on asymptomatic persons vs 22% of mosquitoes feeding on symptomatic persons developed oocysts in their guts [30]. Therefore, reduction in asymptomatic infection is likely to have both individual clinical and public health benefits.

We previously evaluated the impact of CPT on malaria incidence in BAN without restricting the year or season of the clinical event, and found an overall protective effect of CPT on time to incident clinical malaria [5]. However, the protective benefits waned with infant age, similar to our current findings using peak season clinical malaria events. Additionally, CPT was found to be protective against severe clinical malaria (category 3 or 4 serious adverse event) and other severe infant morbidity and mortality outcomes in this study population, with no diminished effect as infants aged [21].

Exposure status was based on time period, with a random review of patient pharmacy records showing good adherence to CPT guidelines [5]. However, as in all studies some exposure misclassification is likely. In addition, we did not account for loss to follow-up and instead used methods that allowed for recurrent malaria events. However, we found a similar association between CPT and clinical malaria as previous BAN analyses that accounted for potentially different follow-up times by CPT exposure status.

Changes in national antimalarial policy increased the use of more effective artemisinin combination therapies for treatment of uncomplicated malaria during the study period. Increase in effective therapy in the community could potentially affect the risk of infection in the later time period (CPT exposed). However, our proxy for transmission (maternal malaria prevalence)

did not indicate a significant or meaningful change in malaria prevalence between the 2 time periods. Last, the limited number of available blood specimens may have biased our sample if infants with and without an available specimen differed by unmeasured variables, such as use of insecticide-treated bed nets. However, infants with and without an available specimen did not differ by any measured baseline covariates in BAN, suggesting that these unmeasured variables are unlikely to have had a significant impact.

Our findings add to the literature detailing the malaria-related benefits of daily CPT use among HEU infants. As WHO guidelines now recommend administration of CPT to all HIV-exposed infants, it would be unethical to conduct a randomized controlled trial of CPT. Thus, retrospective observational studies such as this one are key to evaluating the impact of WHO CPT recommendations. Our findings support the claim that CPT reduces malaria in HEU infants. However, we add important new insights into the impacts of CPT on asymptomatic infection in a setting of high antifolate resistance. As millions of HEU infants are potentially given CPT amid high antifolate resistance, the reductions in asymptomatic parasitemias may reduce malaria transmission and be beneficial for malaria control.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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APPENDIX

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