EFFICACY OF SULFADOXINE-PYRIMETHAMINE IN THE TREATMENT OF UNCOMPROMICATED PLASMODIUM FALCIPARUM MALARIA IN EAST TIMOR

MATTHEW BURNS, JOANNE BAKER, ALYSON M. AULIFF, MICHELLE L. GATTON, MICHAEL D. EDSTEIN, AND QIN CHENG*

Medical Emergency Relief International, London, United Kingdom; Australian Army Malaria Institute, Brisbane, Queensland, Australia; Division of Infectious Diseases and Immunology, Queensland Institute of Medical Research, Brisbane, Queensland, Australia; Australian Centre for International and Tropical Health and Nutrition, University of Queensland, Brisbane, Queensland, Australia

* Address correspondence to Qin Cheng, Department of Drug Resistance and Diagnostics, Australian Army Malaria Institute, Gallipoli Barracks, Enoggera, Queensland 4051, Australia. E-mail: qin.cheng@defence.gov.au

Abstract. The efficacy of sulfadoxine-pyrimethamine (SP) in East Timor is unknown. We treated 38 individuals with uncomplicated Plasmodium falciparum malaria with SP and monitored the outcome for 28 days. Recrudescence parasitemia, confirmed by genotyping, were detected in three individuals resulting in a late treatment failure rate of 7.9% (95% confidence interval = 1.7–21.4%). The results suggest that SP is still efficacious in treating uncomplicated P. falciparum malaria in East Timor. However, the useful life of SP in East Timor may be limited because 80% of the parasites in our samples were found to already carry double mutations in P. falciparum dihydrofolate reductase (S108N/ C59R). The data from this study also highlights that the presence of gametocytes may significantly influence the estimate of SP efficacy determined by genotyping.

INTRODUCTION

Chloroquine was the first-line therapy for uncomplicated Plasmodium falciparum malaria in East Timor prior to the acute complex emergency situation in 1999. In 2000, it was reported that chloroquine efficacy was poor in a hyperendemic area of East Timor (Lautem District), where two-thirds of the sample population treated with chloroquine experienced recurrent parasitemia.¹ ² As a result, sulfadoxine-pyrimethamine (SP) was introduced as the first-line therapy on a national level within East Timor in 2000. Since resistance to SP is widespread in southeast Asia, South America, and West Papua Province of Indonesia, there is an urgent need to determine the extent of SP resistance in East Timor.

Sulfadoxine-pyrimethamine inhibits the parasite folate synthesis pathway by targeting dihydropteroate synthase (DHPS)³ and dihydrofolate reductase (DHFR).⁴ It is well established that mutations in parasite DHPS are responsible for resistance to sulfadoxine,⁵ while mutations in parasite DHFR are responsible for resistance to pyrimethamine.⁶ ⁹ Generally, the number of mutations accumulated in these parasite molecules relates to the level of resistance to SP.¹⁰ Therefore, determination of the prevalence and number of mutations in DHFR and DHPS in a parasite population helps in the assessment of drug selection pressure and the development of resistance. We report the first SP efficacy trial conducted in Los Palos in the Lautem District of East Timor. We also examined the prevalence and the number of genetic mutations in dhfr and dhps in P. falciparum isolates obtained from the infected patients as a means of determining the likely usefulness of SP in East Timor.

MATERIALS AND METHODS

Study area. The study was carried out at the Central Los Palos health post in the Lautem district of the east sector of East Timor (8°30’S, 127°00’E, altitude = 393 meters). Patients attending the facility mainly reside in the sub-districts of Los Palos and Lautem.

Patient recruitment. An in vivo efficacy study was conducted using World Health Organization (WHO) guidelines,¹¹ with slight modifications to include patients with a lower upper limit of parasitemia and a history of fever within the last 12 hours. A sample size of 42 was calculated according to the double lot quality assurance method as recommended by the WHO,¹¹ taking an estimated prevalence of 25% treatment failure as indication of replacement of the first-line antimalarial drug, a 10% failure rate as acceptable with a power of 80% and a 95% confidence interval (CI). The protocol was reviewed and approved by the Division of Health Services (currently Ministry of Health) and by the WHO in East Timor. Potential participants attending the outpatient facility from March to August 2001 were initially screened with a malaria rapid diagnostic test (RDT) specific for the histidine-rich protein 2 antigen of P. falciparum. Those testing positive were further tested by confirmatory microscopy using Giemsa-stained blood slides. Patients having a P. falciparum mono-infection with a patent asexual parasitemia of 1,000–30,000/μL were then examined clinically.

Patients were included in the study if they were greater than six months of age, and had an axillary temperature ≥ 37.5°C but less than 39.5°C, or a history of fever within the last 12 hours. Informed consent was obtained from the patient or parent/guardian if the patient was less than 18 years of age. Patients were excluded if they had severe disease or one or more of the general danger signs of severe malaria, including a hemoglobin level < 5 g/dL, if they were pregnant or had a febrile disease other than malaria, or if they had a history of hypersensitivity or skin conditions to sulfonamides or any other drugs.

Patient treatment and follow-up. Patients were weighed and given a supervised oral dose of SP (sulfadoxine, 25 mg/kg and pyrimethamine, 1.25 mg/kg). Patients remained in the clinic under observation for one hour after each dose. Paracetamol was given to reduce fever and the patient or parent/guardian was instructed to use tepid sponging for the initial 24–48 hours because of the non-apyretic effect of SP. Parasitologic and clinical follow-up was conducted on days 1, 3, 7, 14, 21, and 28, and on any other day the patient had recurring...
symptoms. Blood samples (4 mL) were taken prior to treatment and on days 1 and 28 post-treatment, and when treatment failure was observed. These samples were used for hemoglobin analysis using an electronic photometer (HemoCue Inc., Lake Forest, CA), molecular analysis, and analysis of blood SP concentrations. Blood samples collected for molecular analysis were stored in 6 M guanidine solution. For drug analysis, blood samples were stored at approximately −15°C initially and then transferred on dry ice to the laboratory and stored at −80°C until analysis.

Treatment failures with SP were treated with oral quinine sulfate (10 mg/kg, three times a day for seven days). Patients treated with quinine were asked to attend the facility if they experienced illness or fever over the next 28 days, and were reviewed on day 14 for clinical and parasitologic assessment. Patients with clinically severe disease were treated appropriately and withdrawn from the study. Patients with mild intercurrent illness treated with drugs having no antimalarial activity remained in the study.

**Microscopic examination of blood smears.** Thick and thin blood smears prepared on days 0, 3, 7, 14, 21, and 28 post-treatment were stained with Giemsa and microscopically examined. Approximately 200 oil-immersion fields were examined for each smear and asexual and sexual parasite densities were quantified against 200–500 white blood cells.

**Classification of treatment outcomes.** Treatment outcomes were categorized according to WHO guidelines as an adequate clinical and parasitologic response (ACPR), early treatment failure (ETF), and late treatment failure (LTF) including late clinical failure (LCF) and late parasitologic failure (LPF).

**Detection of parasite DNA and genotyping.** Merozoite surface proteins 1 and 2 (msp-1 and msp-2) were amplified to determine the number and type of alleles in each sample. Pre-treatment samples were genotyped to determine the degree of diversity of the two markers and allele distribution in the sample set. To distinguish recrudescence from new infections, genotyping was performed for paired pre-treatment and failure samples. To assess whether there were potential LPFs that may have been missed by microscopic examination, all samples of individuals classified as ACPR were also genotyped. Extraction of DNA from blood stored in guanidine was performed using the Wizard Plus Miniprep DNA purification system (Promega, Madison, WI) in accordance with the manufacturer’s instructions. Both msp-1 and msp-2 were amplified, and the measured product size was used to assign allelic types to the parasites. We defined recrudescence as treatment failure with parasites having an identical allelic type to the pre-treatment sample, while a new infection was defined when parasites detected in post-treatment samples had a different allelic type than the pre-treatment sample.

**Determination of genetic mutations in P. falciparum dhfr and dhps.** Portions of the P. falciparum dhfr and dhps genes were amplified from all pre-treatment samples, any sample from a treatment failure, and any sample with detectable parasite DNA on day 28 using a nested polymerase chain reaction (PCR). For dhfr, the first-round PCR was performed in a standard 50-µL reaction mixture using primers DHFR1 (5′-TTTATATTTTCCCTTTTG-3′) and DHFR2 (5′-CATTATTATTTGTTTCTT-3′) and the following cycling conditions: 10 minutes at 94°C followed by 40 cycles of denaturation at 94°C for 50 seconds, annealing at 42°C for 50 seconds, and elongation at 70°C for 1 minute. A second-round PCR was performed using primers DHFR3 (5′-TTTATGATGGAAACAGTTGCTTCT-3′) and DHFR4 (5′-TTTACTGATATACTGCTAAACAG-3′) and the cycling conditions described for the first round except that the annealing temperature was 50°C. The PCR product was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) and then sequenced. Nucleotide sequences were translated into amino acid sequences and amino acid mutations were determined.

For dhps, the first-round amplification was performed using primers DHPS1 (5′-TGATACCCGAATATAAGCATAAAT-3′) and DHPS2 (5′-ATAATAGCTGTTAGGAAGCAATTG-3′), and the second-round amplification was performed using primers DHPS3 (5′-ATGATAATGAAGGTGCTGTAGT-3′) and DHPS4 (5′-CAATTGTTGTGATTGTCCAC-3′). The PCR conditions for the first and second rounds were identical to those used in dhfr amplifications. The product was digested with Ava II for identification of mutations at position 437 using the methods reported by Duraisingh and others. A random sample (n = 10) of the PCR products for dhps was then sequenced to confirm the result.

**Measurement of blood SP concentrations.** Blood SP concentrations were measured by high-performance liquid chromatography. The inter-assay coefficients of variation for sulfadoxine and pyrimethamine were 1.1% and 7.2%, respectively, at corresponding concentrations of 0.5 µg/mL and 50 ng/mL (n = 5), with an inaccuracy of < 7% for both drugs. The limit of detection was 0.1 µg/mL for sulfadoxine and 10 ng/mL for pyrimethamine.

**Statistical analysis.** Differences in the distribution functions for the parasitemia on the day of treatment, age, and SP concentrations at one day post-treatment between patients with LTF and ACPR were compared by using the Mann-Whitney U test. This test was also used to compare the SP concentrations one day post-treatment of patients who were PCR positive and PCR negative on day 28. Forward binary logistic regression was used to assess the influence of gametocyte density on the PCR positivity of samples obtained 28 days post-treatment.

**RESULTS**

**Patient recruitment.** Forty-five patients met the inclusion criteria for enrollment. Seven subjects were subsequently excluded from the study because of infections with P. vivax (n = 3), mixed infection with P. vivax (n = 3), and loss to follow-up (n = 1). The remaining 38 patients (21 males and 17 females) were treated with SP and followed-up for 28 days. Twenty-one of these 21 patients reported previous malaria infections. The median age of patients was 17 years (range = 2.5–65). Approximately, 38% of the patients were between 6 and 15 years of age.

Of the 38 patients, 6 reported having been previously treated with chloroquine and 1 patient had taken chloroquine on the day prior to SP treatment. Twenty-seven patients had symptoms of uncomplicated P. falciparum malaria, while the remaining 11 were asymptomatic at the time of presentation but had a positive RDT result confirmed by microscopy and a history of fever within the previous 12 hours. Parasitemias of the 38 patients are shown in Figure 1. Infection with P.
falciparum was confirmed in each of the 38 patients by PCR amplification of *msp-1*, *msp-2*, *dhfr*, and *dhps*.

**Clinical and parasitologic response to treatment.** During the 28 day follow-up period, we observed 3 patients with microscopy-detectable *P. falciparum*: 2 patients on day 7 (1 LCF and 1 LPF) post-treatment and 1 patient on day 28 (LPF). The remaining patients were categorized as an ACPR. This gave an overall treatment failure rate of 7.9% (95% CI = 1.7–21.4%). All 3 LTFs were subsequently treated effectively with quinine.

Most patients (n = 33, 86.8%) were initially responsive to SP treatment because parasitemia cleared by day 3. Five patients showed low-grade *P. falciparum* infections (< 0.02% of the starting parasitemia) on day 3, of which 3 cleared parasites and 2 remained parasitemic at day 7. Symptomatic improvement and clearance of fever paralleled the clearance of parasitemia; fever resolution on day 3 post-treatment was 95% (n = 36). One patient who did not show symptomatic improvement was parasiticemic on day 7. Thirteen (32.5%) patients had hemoglobin levels < 10.0 g/dL on day 0, but by day 28 or on the day of treatment failure their hemoglobin had increased above this level. Only 2 patients (5%) continued to have hemoglobin levels < 10g/dL, of which 1 was an LTF. There was no significant difference in the starting parasitemia between the 3 LTF cases (median = 10,627 parasites/µL, range = 1,600–25,441) and the successfully treated individuals (median = 7,159 parasites/µL, range = 1,100–25,537) (P > 0.05). The median age of the 3 LTFs was 24 years (range = 7–51) and was not significantly different from that of the successfully treated group (17 years, range = 2.5–65) (P > 0.05).

**Genetic diversity, allelic distribution, and multi-clonal infections.** Genotyping of the 38 pre-treatment samples using *P. falciparum* *msp-1* and *msp-2* identified 7 allelic types of *msp-1* and 15 allelic types of *msp-2*. The combination of *msp-1* and *msp-2* allelic types resulted in a minimum of 36 distinct allelic types in the 38 samples, of which 22 types were only seen once (Figure 2). There was no apparent dominance of any allelic type in the pre-treatment or post treatment samples (Figure 2). Mixed allelic types (multi-clone infection) were detected in 42% (16 of 38) of the pre-treatment samples with a maximum of 3 allelic types in 1 patient. Interestingly, all 3 LTF cases had single clone infections when recruited, while only 54% (19 of 35) of the remaining patients had single clone infections.

**Allelic types in the paired pre-treatment and post-treatment samples.** An identical allelic type was detected in the paired pre-treatment and post-treatment samples for the 3 LTFs confirming their recrudescence of infection post-treatment. Genotyping was also performed on day 28 samples for the remaining 35 ACPRs to examine whether there were any LPFs that may have been missed by microscopic exami-
nation. Parasite DNA was not detected by PCR in 14 patient samples on day 28, which confirming the cures. Of the 21 remaining day 28 samples, differing allelic types were detected in 6 pairs of samples (15.8%) including 3 types not seen in any pre-treatment samples. The remaining 15 patient samples, which were positive by PCR, had identical allelic types in their pre-treatment and post-treatment samples.

**Influence of gametocytemia on PCR genotyping.** Gametocytes were seen in 19 of 35 ACPR samples on day 28, with densities ranging from 38 to 1,388 gametocytes/μL. The PCR analysis detected parasite DNA in 17 of 19 gametocyte-positive samples, but failed to detect DNA in the 2 remaining samples, which had relatively low gametocyte counts (38/μL). Logistic regression analysis of 29 samples collected on day 28 (exclude LTF and new infections) indicated that the density of gametocytes was predictive of PCR positivity (P < 0.0002, Nagelkerke R² = 0.51), with densities greater than 18.8 per μL predicting a positive PCR result (Figure 3A). The overall accuracy of the prediction was 79%. Decreasing gametocytemias were observed in 18 of 19 individuals from days 14 to 28 (Figure 3B).

**Blood SP concentrations.** The median blood sulfadoxine and pyrimethamine concentrations in patients with an adequate response were 99.65 μg/mL (range = 72.8–155.3) and 278 ng/mL (range = 106–468 ng/mL), respectively, on day 1 after SP administration. By day 28 post-treatment the mean sulfadoxine concentration had decreased to 5.81 μg/mL (range = 0.6–18.1), with pyrimethamine concentrations below the limit of detection (10 ng/mL). The 3 LTFs had blood sulfadoxine (median = 104 μg/mL, range = 97–111) and pyrimethamine (median = 245 ng/mL, range = 120–368) concentrations 1 day post-treatment that were not statistically different from those in those patients having an adequate response (P > 0.05). At the time of their failures they had individual sulfadoxine and pyrimethamine concentrations of 69 μg/mL and 127 ng/mL (day 7), 66 μg/mL and 62 ng/mL (day 7), and 0.35 μg/mL and < 10 ng/mL (day 28), respectively. Only one patient had a measurable sulfadoxine concentration (0.33 μg/mL) before treatment.

**Mutations in dhfr and dhps.** Of the 38 pre-treatment samples, 7 (18.4%) had the wild-type dhfr sequence, while the remaining 31 sequences (81.6%) had single or double mutations in dhfr: 1 with S108N and 30 with C59R/S108N. *Plasmodium falciparum dhps* was amplified and the mutation at 437 was investigated by restriction fragment length polymorphism analysis. None of the isolates were digested with Ava II, which suggested that all were wild-type dhps sequences. A random selection of 10 samples were sequenced and confirming the wild-type sequences at amino acid positions 436, 437, 540, 581, and 613 in all samples. All 3 LTFs (100%), along with five of the six new infections (83%) and 23 of the 29 cases (80%) who responded adequately to SP were infected with parasites with double mutations in dhfr.

**DISCUSSION**

The present study was the first SP efficacy trial for the treatment of uncomplicated *P. falciparum* malaria conducted in East Timor since the introduction of this drug combination as the country’s first-line anti-malarial treatment drug one year earlier. Based on ACPRs, the SP cure rate for the 38 patients during the 28-day follow-up was 92.1%. To obtain an accurate failure rate, PCR-based genotyping was used to detect sub-microscopic parasitemia and to distinguish recrudescent from new infections. Genotyping results showed diversity in the parasite population with at least 36 distinct allelic types detected in the 38 patient samples. This number and the non-dominant distribution of allelic types indicate a large parasite reservoir in the study area, which resulted in a low probability of an individual being infected with the same allelic type. In addition to confirming 3 LTFs, genotyping de-
ected parasite DNA in the day 28 samples from an additional 22 individuals. Six of these 22 individuals had genetically different parasites compared with their pre-treatment samples, indicating either new infections or a selection of subpopulations that have survived residual SP. Five of the six new parasites had double mutations in their DHFR, indicating a resistance level capable of surviving residual SP concentrations. The remaining 16 individuals had identical parasite allelic types in their pre-treatment and post-treatment samples. If these patients are considered treatment failures, the overall SP failure rate would increase to 50%.

There are two possible explanations for the difference between the number of clinical failures and patients with parasite DNA. First, the PCR may have detected submicroscopic asexual parasitemia, which would have developed into patent parasitemia if the follow-up period had been longer. Second, the DNA detected by the PCR originated from residual gametocytes in the patients' samples. Several studies have reported an increase in gametocyte prevalence and density after SP treatment compared with chloroquine,16–18 and it has been suggested that gametocytes present after treatment result from drug-selected asexual parasites.20 Concern has been reported over the relationship between persistent gametocytes and positive PCR results,21,22 however, the extent of the influence of gametocytes after SP treatment on PCR results is not clear. Although our results do not directly show that the presence of gametocytes resulted in positive PCR detection of identical allelic types, the presence of gametocytes was predictive of a positive PCR result on day 28. A reverse transcription–PCR assay detecting gametocyte and asexual stage specific transcripts may need to be used in future to quantify the presence of gametocytes and submicroscopic asexual parasites. Thus, by incorporating both explanations, and considering that approximately 50% of the patients recruited were adults with moderate parasitemias, both of which may lead to an underestimation of failure rate, we believe that the true failure rate for this study lies between 7.9% and 50%. Further studies in different areas will provide a nationwide assessment on SP efficacy in East Timor.

It is well established that mutations in parasite DHFR and DHPS are responsible for resistance to SP.7–9 In the dhfr gene, the mutation S108N has been identified as a primary requirement for pyrimethamine resistance, and the combination of S108N/N51I or S108N/C59R is often seen as the second stage of the resistance progression. In this study, 81.6% of the parasites carried mutations in dhfr with most (78.9%) having the double mutation S108N/C59R, while none had mutations in dhps. This is consistent with asymmetric selection observed for point mutations in dhfr and dhps, in which mutations in dhfr usually occur first, followed by mutations in dhps.10 Unlike the parasite genotype in Uganda,7 but similar to that in Kenya23 and Papua New Guinea,25 C59R appears to have been selected prior to N51I in East Timor. Compared with southeast Asia, South America, and Africa where mutant DHFR alleles with triple mutations combined with double mutations in DHPS are common, the level of mutations in East Timor appears low, which is consistent with the observed low failure rate. However, if one considers that SP had only been introduced one year earlier in East Timor, and that its usage is not widespread, this level of resistance mutation, particularly double mutations, is fairly high. It is probable that these resistant parasites have spread from other countries as has been shown for other regions of the world.26,27

If one considers the rapid selection of SP resistance in Africa and South America and takes into account that East Timor has extensive population movement (primarily from West Timor), widespread heterogeneity of transmission intensity, and uncertainties over sub-optimal drug use (especially in private sectors in sub-districts), which are factors known to be associated with drug resistance, it might be expected that the useful life span of SP is limited in this country. Thus, alternative affordable antimalarial drug combinations for the treatment of malaria should be considered for East Timor in preparation for the time when SP resistance develops to unacceptable levels.

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Authors’ addresses: Matthew Burns, Centre for International Emergency, Disaster and Refugee Studies, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205. E-mails: simulium98@yahoo.com and mburns@jhsph.edu. Joanne Baker, Alison M. Auliff, Michael D. Edstein, and Qin Cheng, Australian Army Malaria Institute, Weary Dunlop Drive, Gallipoli Barracks, Enoggera, Brisbane, Queensland 4051, Australia, Telephone: 61-7-3332-4801, Fax: 61-7-3332-4800, E-mails: Joanne.bakeri@defence.gov.au, alison.auiliff@defence.gov.au, mike.edstein@defence.gov.au, and qin.cheng@defence.gov.au. Michelle L. Gatton, Malaria Drug Resistance and Chemotherapy, Division of Infectious Diseases and Immunology, Queensland Institute of Medical Research; Australian Centre for International and Tropical Health and Nutrition, University of Queensland, Post Office Royal Brisbane Hospital, Brisbane, Queensland 4029, Australia, Telephone: 61-7-3362-0416, Fax: 61-7-3362-0104, E-mail: michelle.gatton@qimr.edu.au.

REFERENCES


