Efficacy of Chloroquine for the Treatment of Uncomplicated Plasmodium falciparum Malaria in Honduras

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INTRODUCTION

Development of resistance to chloroquine (CQ) and other drug treatment in Plasmodium falciparum malaria has led the World Health Organization (WHO) to change its recommendations to adopt artemisinin combination therapy (ACT) as the first-line drug for the treatment of P. falciparum malaria cases in most endemic countries. In the Americas, CQ-resistant P. falciparum has been documented in all South American countries endemic for malaria since the 1950s, including Brazil, Bolivia, Colombia, Ecuador, Peru, Guyana, Suriname, and Venezuela. All of these countries have changed their drug policies in alignment with WHO recommendations to adopt ACT. In contrast, for Central American countries (except Panama) and the island of Hispaniola, CQ continues to be the first line of treatment of P. falciparum malaria, because there is no evidence for the presence of CQ resistance in Central America outside of Panama, Mexico, or the Caribbean islands. Panama is the only Central American country with documented CQ-resistant P. falciparum malaria and as a result, changed its first line of treatment to sulfadoxine-pyrimethamine (SP) combination in 2004.

Honduras has the highest malaria burden in Central America. Honduras accounted for 69%, 84%, and 91% of all P. falciparum cases reported in Central America in 2007, 2008, and 2009, respectively. A remarkable decrease in the transmission of the disease has been observed in the country in the last decade: from 35,125 cases in 2000 to 8,368 cases by 2008. Most of the malaria cases are found in the northeastern region of the country, particularly the state of Gracias a Dios, which reported 33% (2,798 cases) of all malaria cases in 2008 and accounted for 69–94% of P. falciparum cases between 2007–2009.

In Honduras, CQ efficacy was determined using in vivo protocol only in 1981, and since that time, no additional studies have been conducted. To confirm the efficacy of CQ for the treatment of P. falciparum malaria in Honduras, an in vivo study was conducted using Pan American Health Organization (PAHO) – WHO protocol with a 28-day follow-up. In addition, P. falciparum isolates collected from this study were tested for the presence of CQ-associated molecular markers in the pfcrt gene.

MATERIALS AND METHODS

Study location and patient enrolment. The study was carried out from September of 2008 to September of 2009 in the municipality of Puerto Lempira, Gracias a Dios, Honduras (Figure 1), a highly endemic area for P. falciparum malaria. This study was approved by the institutional ethical review committee of the Medical Sciences Faculty of the National Autonomous University of Honduras (UNAH-IRB 0003070). All participants provided written consent to participate in the study. Patients residing within a radius of 30–45 minutes of travel time (car or boat) were included, because this area was convenient for the study team to conduct follow-up observations. Criteria for patient inclusion were uncomplicated P. falciparum malaria as described by national and international definitions. This criteria included participants aged between 6 months and 60 years with microscopically detectable mono-infection with P. falciparum, parasitemia levels greater than 250 but less than 50,000 asexual forms/μL, willingness to attend follow-up visits for 28 days, and willingness to participate in the study and provide informed written consent. The exclusion criteria included pregnancy or lactation (pregnancy test was performed for women of childbearing age), signs of severe malaria, underlying chronic disease (cardiac, renal, or hepatic disease or malnutrition), history of allergy to CQ, and presence of mixed infection with different species of malaria parasites.

Sample size. The sample size was determined according to the proportion of treatment failures expected in this population. Assuming a CQ treatment failure rate of 5% in a population of infinite size, a power of 80%, and a significance level of 5%, 42 patients per treatment would be required. If allowing for 15% attrition, 49 patients would be needed. A total of 69 patients who were determined to have only P. falciparum infection based

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on microscopic diagnosis were enrolled for this study, and 68 of the patients completed the follow-up until day 28.

**Microscopy and sample collection.** Blood smears were stained with 3% Giemsa for 30 minutes at room temperature. Smears were analyzed by experienced microscopists. The parasite density was determined by using the PAHO–WHO criteria assuming 6,000 leukocytes/μL. A total of 137 (as one failed to complete follow-up) blood spots (Whatman number 3), including 69 samples collected at the time of enrolment and 68 samples collected at the 28-day follow-up, was dried and stored at −20°C until use for molecular analysis.

**Drug treatment and follow-up.** The antimalarial drug and treatment schedule used was CQ at 25 mg/kg body weight divided into daily doses over 3 days: 10 mg/kg on day 1, 10 mg/kg on day 2, and 5 mg/kg on day 3. SP and quinine were chosen as alternative drugs if CQ was not effective. Supervised treatment was administered to all patients followed by clinical and parasitological evaluation on days 0, 1, 2, 3, 7, 14, 21, and 28. Therapeutic response was classified in patients who completed the follow-up using the nested PCR approach. The primary PCR was performed with 5′-AGCAAAAATGAGCAACGTATAG-3′ (F) and 5′-ATGTGATGATGAAATAGATTCTC-3′ (R) primers with the following cycling parameters: initial denaturation at 94°C for 10 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 30 seconds followed by a final extension at 72°C for 10 minutes. Secondary PCR was done using 5′-TTTTTCCC-TTGTCCACCTTAAC-3′ (F) and 5′-AGGAATAACAA-TAAGAACATAATCATAC-3′ (R) primers. The cycling parameters for secondary PCR were the same as for the primary PCR, except that annealing was set at 56°C for 30 seconds and the number of cycles was reduced to 30. Sequencing of gene fragments was carried out on both strands with their respective nested primers using the standard sequencing protocols on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA) as described earlier.

**Statistics.** Double entry of data was executed and then analyzed using Excel. Median and geometric means were used for analysis of continuous variables and percentages for categorical and nominal variables.

**RESULTS**

A total of 4,827 febrile patients attended two Health Centers, of whom 3,714 patients were negative for malaria according to light microscopy (Figure 2). These patients were referred to medical consultation to investigate other diseases; 1,113 patients were diagnosed with malaria, of whom 791 (71%) patients had *P. vivax*, 263 (24%) patients had *P. falciparum*, and 59 (5%) patients showed a mixed infection with both species. Of the total cases of *P. falciparum* malaria captured in the sentinel sites, only 69 cases were included in this study (26%); the remainder of cases did not meet protocol inclusion criteria or the patient refused to participate. Among these 69 patients included in the study, 1 patient was excluded from the final analysis, because this patient was found to have only *P. vivax* but not *P. falciparum* infection based on subsequent PCR test.

Demographic data of study participants are given in Table 1. All patients selected for the study were clinically evaluated according to height, weight, physical examination, and blood pressure on day 0, whereas temperature and blood smear examination were done on days 0, 1, 2, 3, 7, 14, 21, and 28 (Figure 3). At the end of evaluations on day 7, all patients were parasitemia-negative (absence of microscopically detectable asexual stages in blood), and none developed parasitemia through day 28 of the trial, indicating 100% efficacy of CQ (Figure 4). None of the patients reported having taken antimalarial drugs on their own (CQ, primaquine, or SP); 57 patients (95%) took analgesic, antipyretic, and/or anti-inflammatory medications to reduce symptoms and discomfort.

**Molecular markers associated with CQ resistance.** The *pfcr* gene sequencing data for 68 samples showed no mutations within amino acid residues 72–76. All the samples had the nested polymerase chain reaction (PCR) was performed with primers and cycling conditions as described by Singh and others. PCR results confirmed presence of *P. falciparum* infection in 68 patients, and 1 patient was found to have *P. vivax* but not *P. falciparum* infection as reported recently.

To determine if there is any parasite with CQ-resistant genotype, the DNA was subjected to the PCR amplification of the partial regions of *pfcr* (covering codons 72–76) using a nested PCR approach. The primary PCR was performed with 5′-AGCAAAAATGAGCAACGTATAG-3′ (F) and 5′-ATGTGATGATGAAATAGATTCTC-3′ (R) primers with the following cycling parameters: initial denaturation at 94°C for 10 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 30 seconds followed by a final extension at 72°C for 10 minutes. Secondary PCR was done using 5′-TTTTTCCC-TTGTCCACCTTAAC-3′ (F) and 5′-AGGAATAACAA-TAAGAACATAATCATAC-3′ (R) primers. The cycling parameters for secondary PCR were the same as for the primary PCR, except that annealing was set at 56°C for 30 seconds and the number of cycles was reduced to 30. Sequencing of gene fragments was carried out on both strands with their respective nested primers using the standard sequencing protocols on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA) as described earlier.
CVMNK amino acid sequence (CQ-sensitive ancestral genotype) in this region, confirming the absence of parasites with CQ-resistant pfcr allele.

DISCUSSION

The emergence of drug-resistant strains of *P. falciparum* has contributed to worldwide resurgence of malaria in recent decades, and it is associated with increased mortality and morbidity. Drug susceptibility usually relies on various factors, such as the intensity of infection, immune status, plasma concentrations of the drug, and duration of drug application; however, the inherent capacity of the parasite to tolerate drugs is mostly based on its genotype. We conducted this study to evaluate *in vivo* effectiveness of CQ against *P. falciparum* malaria infections and determine the pfcr genotypes of the *P. falciparum* strains currently circulating in a highly endemic region of Honduras.

Although Honduras has not reported resistance to the current first-line antimalarial drugs CQ and primaquine, it remained unclear if the efficacy of CQ has changed over the time, and there have been occasional local anecdotal reports of treatment failure by both physicians and patients. Another concern was whether any potential misuse of CQ in the local population has altered the clinical efficacy of this drug. Recently, the state of Gracias a Dios has become a major hub for human migration from South America, where CQ resistance is fixed in *P. falciparum* parasites. This finding has

**Table 1**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N = 68*</th>
</tr>
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<tbody>
<tr>
<td>Median age in years (range)</td>
<td>17 (1–40)</td>
</tr>
<tr>
<td>Children under 5 years (%)</td>
<td>7, 10</td>
</tr>
<tr>
<td>Children 5–15 years (%)</td>
<td>21, 31</td>
</tr>
<tr>
<td>Adults</td>
<td>40, 59</td>
</tr>
<tr>
<td>Males (%)</td>
<td>39, 57</td>
</tr>
<tr>
<td>Axillary temperature ≥ 37.5°C (day 0; %)</td>
<td>46</td>
</tr>
<tr>
<td>Geometric mean parasite density (μL⁻¹; day 0)</td>
<td>6,268</td>
</tr>
</tbody>
</table>

*Although 69 patients were enrolled based on the microscopic diagnosis of *P. falciparum*, 1 patient was excluded from the final analysis, because subsequent PCR experiments determined presence of *P. vivax* infection but not *P. falciparum* infection in that patient.

**Figure 2.** Flowchart of the enrolment process for *in vivo* drug efficacy study based on microscopic diagnosis in Puerto Lempira, Honduras.

**Figure 3.** Number of study participants with fever (> 37.5°C) by day of observation in the *in vivo* drug efficacy study in Puerto Lempira, Honduras.
raised concern about potential importation of CQ-resistant parasites through human migrants. Therefore, continuous monitoring for the CQ efficacy and molecular surveillance for CQ-resistant pfcr1 genotype in this region are essential for the support of current drug policy.

Because there are different parameters of clinical and parasitological assessment to evaluate resistance to antimalarial drugs, standard protocols were used in this study.29 We used the PAHO–WHO standard methodology and followed patients for 28 days to assess the efficacy of CQ for the treatment of uncomplicated P. falciparum infection. In this study, the most important finding is the 100% efficacy of CQ treatment of clearing P. falciparum infection during the 28 days of follow-up. This finding is further confirmed by the exclusive presence of only CQ-sensitive ancestral codons of the pfcr gene (CVMNK genotype) in all the parasite samples tested. This study is consistent with the research conducted 3 decades ago,15 which showed, at the regional level, the usefulness of CQ as a first-line drug. Our study provides strong support for the continuation of CQ for the primary treatment of malaria in Honduras for now.

This study was carried out in the area with the highest incidence of malaria in Honduras and did not include patients from other regions of the country. Although the data from this study cannot be extrapolated to the rest of the country, recent results obtained by Jovel and others30 confirm that P. falciparum isolates from other regions of the country also possessed a susceptible wild-type pfcr1 genotype. Furthermore, they showed that CQ-resistant alleles of pfmdr1, sulfadoxine-resistant pfldhs alleles, and pyrimethamine-resistant pfldhr alleles were also not detectable in this region. Because conducting in vivo drug efficacy trials can be expensive, it is reasonable to continuously monitor the parasite populations from different parts of the country for the presence of CQ-resistant pfcr1 allele as an early warning signal for the potential emergence of CQ resistance.

The findings from this study are consistent with recent reports indicating that CQ remains efficacious for the treatment of falciparum malaria in Nicaragua31 and Haiti.32 Collectively, these findings are consistent with continuation of CQ as the primary drug of choice for the treatment of malaria in this region. It is still unknown why CQ-resistant parasites have failed to establish in Central American regions outside of Panama, although CQ-resistant parasites are fixed in South America.11 Implementation of an active molecular surveillance program to detect emergence of any CQ-resistant P. falciparum parasites will help to develop appropriate measures to prevent the spread of resistant parasites and evaluate the efficacy of CQ for continued treatment of falciparum malaria in this region.

In conclusion, this study showed evidence that there is no resistance to CQ, which is the first-line drug used in the country, according to the National Guidelines for malaria.10 However, it is necessary to maintain regular monitoring of therapeutic efficacy of antimalarial drugs with standardized methodology and rigorous quality control. An early indicator is the presence of CQ-resistant molecular markers, which can be monitored through the application of routine molecular surveillance of P. falciparum isolates in the country.

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![Figure 4. Geometric means of asexual parasite density by day of observation in the in vivo drug efficacy study in Puerto Lempira, Honduras.](image-url)


