

Therapeutic Efficacy of Artesunate-Amodiaquine and Polymorphism of *Plasmodium falciparum* k13-Propeller Gene in Pala (Tchad)

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Abstract

ACTs was recommended as a first-line treatment for uncomplicated *Plasmodium falciparum* malaria in many malaria-endemic countries. Regular monitoring of ACTs is recommended by the World Health Organization (WHO) to help early detection of resistant parasite strains and contain their rapid spread. The aim of this study was to assess therapeutic efficacy of Artesunate-Amodiaquine (ASAQ) the first line treatment of uncomplicated falciparum malaria in Chad and analyze the polymorphism of Kelch13-propeller gene.

A single-arm prospective study of a 28-days follow-up was conducted among children aged 6- 59 months with uncomplicated *P. falciparum* malaria at Pala site from November to December 2015. The primary outcome was ACPR PCR-corrected at day28 and the secondary endpoints were the Parasite Clearance Time (PCT), Fever Clearance Time (FCT) and tolerability of the drug Kelch13-propeller was amplified and sequenced in all *Plasmodium falciparum* isolates.

A total of 58 children were enrolled and 51 reached the study endpoint. Crude Adequate and clinical response was 98% at day 28 and after PCR correction this rate was 100%. Treatment was well tolerated. No mutations neither synonymous nor non synonymous were detected on k13 gene, after alignment with the reference sequence PF3D7_1343700.

ASAQ was proved to be efficacious and well tolerated in Pala children and no mutation was observed in the Kelch 13-propeller gene. Further studies are needed across the country to enhance resistance surveillance.

Key Word: Malaria, *P. falciparum*, Artesunate-Amodiaquine, k13-propeller, Chad

Introduction

Malaria remains one of the most important public health challenges in the world. Malaria is the leading cause of morbidity and mortality in Africa, particularly in children under 5 years old and pregnant women. Artemisinin-Based Combination Treatments (ACTs) are now the recommended first-line treatments for uncomplicated falciparum malaria worldwide. The worldwide use of ACTs has contributed in recent years to a substantial reduction in deaths related to *falciparum* malaria. Resistance to artemisinin however has emerged in Southeast Asia [1-5].

Monitoring the efficacy of ACTs becomes particularly important in the light of emergence of artemisinin resistance in South-East Asia [6,5]. Artemether-Lumefantrine (AL) and Artesunate-Amodiaquine (ASAQ) are widely available drugs, which are recommended by most malaria endemic countries in the treatment of uncomplicated *P. falciparum* malaria [7]. Chad National Malaria Control Programm (NMCP) recommends, since 2005, ASAQ and AL respectively as first and second line treatments for uncomplicated *P. falciparum* malaria. The World Health Organization recommends a regular assessment of the efficacy of the first- and second-line antimalarial drugs for an early detection and prevention of the spread of resistant parasite populations [8].

In vivo therapeutic efficacy study is the gold standard for detecting the emergence and spread of malaria drug resistance.

Discovery of mutations in the *Kelch*-13 propeller region protein in correlation with delayed clearance phenotype is a major advance. Surveillance of artemisinin resistance to date relied on *in vivo* studies to measure early clearance of peripheral parasitaemia by light microscopy and K13 propeller gene mutations. Since the introduction of ACTs in 2004 in Chad, very few studies have been conducted on ACTs efficacy and *Kelch*13-propeller gene [9,20]. The aim of this study was to assess the *in vivo* therapeutic efficacy of Artesunate-Amodiaquine (ASAQ) and analyze the polymorphism of the *Kelch* 13 propeller gene conferring artemisinin-resistance to *Plasmodium falciparum* the Pala hospital.

Methods

Study design

This study was a prospective, one-arm assessment of clinical and tolerability of ASAQ according to WHO guidelines [8]. Follow up was for 28 days. The study was conducted at Pala site country in the Mayo-Kebbi west region (9 ° 21'00 "N; 14 ° 58'00" W) of Chad from November to December 2015.

In the study site malaria transmission occurs from July to December during the raining season. The majority of malaria cases in the area is caused by *P. falciparum*, while *Anopheles gambiaes.s.* and to a lesser extent *Anopheles funestus* are the major vectors. The key malaria control interventions in the district include use of LLNIs, malaria case management with ACTs Intermittent Preventive Treatment during pregnancy (IPTp).

To assess the treatment effect on parasites mutations that modulate treatment response, mutation in k13 propeller gene the molecular marker associated with decrease artemisinin sensitivity were investigated [10,11]. The resistance was investigated by examining polymorphisms in the k13 propeller domain at day 0. The method used was nested PCR protocol followed by Sanger Sequencing using primers specific to *P. falciparum*. The amplicon used for sequencing covered 740 pb which included the k13 propeller domain [12].

Study population

Children aged from 6 to 59 months presenting to the facility were enrolled if mono-specific *P. falciparum* infestation was confirmed by microscopy with parasite density between 1000 and 200000 asexual parasites / μ L of blood and they had fever axillary ≥ 37.5 ° C, or history of fever over the last 24 hours. The others inclusion criteria were ability to take oral medications; able to come to come to health facility for follow -up; informed consent of parent or legal guardian. Children with severe malaria symptoms according to the WHO case definition and symptoms of severe malnutrition and chronic diseases or with mixed infection were excluded [8].

Study treatments

Treatment was three-day oral regimen dosed by weight according to the manufacturer's instructions: ASAQ Winthrop® 5 to <9 kg: one tablet/day of Artesunate (AS) 25 mg/Amodiaquine (AQ) 67.5 mg; 9 to <18 kg: one tablet/day of AS 50 mg/AQ 135 mg; 18 to <36 kg: 1 tablet/ day of AS 100 mg/AQ 270 mg.

Children who vomited during the observation period were retreated with the same dose of medicine and observed for an additional 30 minutes. Children with repeated vomiting were excluded and were treated according National Control Program treatment guidelines and excluded from the study. All children were allowed use of antipyretics.

Follow-up procedure

Children enrolled in the trial was followed up for 28 days. Children was seen after the day of enrollement (day 0) on days 1, 2, 3, 7, 14, 21 and 28. At each day visit children were clinically examined by a study physician who recorded findings in a Case Report Form (CRF). Parasitaemia (asexual and sexual) was assessed on days 1, 2, 3, 7, 14, 21, 28 and any day within the 28 days follow-up period that the child is brought to the health facility with fever.

Thick and thin blood smears were stained with 5% Giemsa for 30 minutes. Parasitaemia was determined by reading the thick blood smear and counting the number of asexual parasites per 200 White Blood Cells (WBCs), assuming a WBC count of 8000/ μ l. Slides were considered negative if no parasite was found after reading 100 high-powered fields. Presence of gametocytes was also recorded.

All blood samples were read by two qualified independent microscopist. Slides were quality controlled at the Swiss Tropical Institute and Public Health. Discordance was defined as differences between the first and second microscopist regarding parasite density >50%, species diagnosis or any difference that affected recruitment or study outcome. The first or second reading was taken as final depending on whichever agrees with the third reading.

Filter paper blots were collected at day 0 and at recurrence of parasitaemia for PCR genotyping.

Merozoite surface proteins 1 and 2 (*msp1* & *msp 2*) and Glutamate-Rich Protein (*glurp*) were used to distinguish re-infection and recrudescence.

Safety

At each follow-up visit, any new or worsening symptom was assessed. An adverse event was defined as any unfavorable and unintended sign, symptom or disease temporally associated with the use on investigational product, not present at day 0, but occurred during follow -up, or was present at day 0 but became worse during follow-up. Serious adverse event was defined as any event that resulted in patient hospitalization, death, life-threatening experience, persistent /significant disability or specific medical surgical intervention to prevent serious outcome.

Outcomes

Treatments outcomes were classified based on clinically and parasitological outcomes assessment as recommended by WHO [8]. Therapeutic responses on day 28 were classified as either Adequate Clinical And Parasitological Response (ACPR), or Treatment Failure (TF) designated as Early Treatment Failure

(ETF), Late Clinical Failure (LCF), or Late Parasitological Failure (LPF). The primary outcome endpoint was ACPR, corrected for re-infection using PCR genotyping at day 28.

DNA extraction and PCR

The parasite DNA was extracted from the blood sampled on filter paper on D0 and at failure by a Qiagen DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The k13 gene and msp2 were amplified by a Polymerase Chain Reaction (PCR). For Pfk13 gene, amplified PCR products were sent to GENEWIZ in UK for Sanger sequencing. Subsequent analysis of delivered sequences was executed in comparison with the PF3D7_1343700 sequence. The following co don positions were checked for mutations [10,11].

Statistical analysis

Data management and analysis were completed with Epi Info 6.0.4 adapted to the Who excel-based applications [7]. BioEdit was used for sequences analysis and GraphPad Prism 5 (one way ANOVA test) was used to compare the 3 mean temperatures from Day 0 to Day 2.

Results

Profile of Study Patients

During the study period from November to December 2015, 163 patients were examined for uncompleted malaria at the Urban Health Center in Pala. Among them, 58 were randomized and 105 were excluded from the study for the reasons detailed on the study profile (Figure 1).

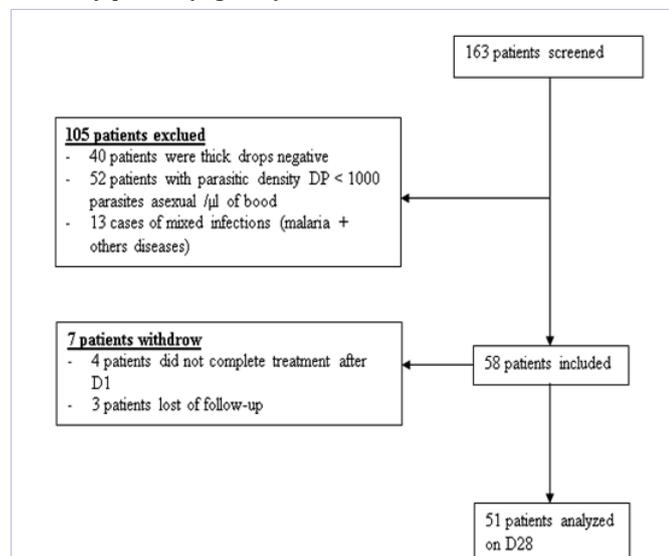


Figure 1: Study profile

Characteristics of the study

Of the 58 randomized patients, 32 (30.5%) were female and 26 (24.8%) were male, with a sex ratio of 0.81; Mean age (ds) 2.9 (1.3%), average weight (ds) 13.2 (3.6%), average temperature (ds) 38 (0.9%) and mean parasite density of 3.840 asexual parasites/µL on the day of enrolment (Table 1).

Table 1: Baselines characteristics of the study

Parameters	Items
Screened	163
Included n (%)	58 (35.6 %)
Male n (%)	26 (44.8 %)
Female n (%)	32 (55.2 %)
Sex ratio	0.81
59 months n (%)	8 (13.8 %)
< 59 months n (%)	50 (86.2 %)
Mean age (± SD) years	2.9 (±1.3)
Age (min - max) years	0.7 - 5
Mean weight (± SD) kg	13.2 (± 3.6)
Weight (min - max) kg	5 - 21
Mean axillary temperature (± SD) ° C	38 (± 0.9)
Axillary temperature (min - max) ° C	36.2 - 40.3
Mean parasitemia (asexual parasites / µL)	3840
Parasitemia (min - max) asexual parasites / µL	1.040 - 20.000

Therapeutic efficacy

A total of 58 patients were included and 51 patients were analyzed per protocol. Prior to PCR correction, 50 patients were ACPR that is 98. % treatment success with only one (2.0 %) Late Clinical Failure. After PCR correction, the failure reported on Day 28 was in fact a re-infestation. Thus, all treated patients were 100 % ACPR on Days 28 (Table 2).

Table 2: Therapeutic response at D28

	Per-protocol analysis
Patients screened at D28	51 (87.9%)
Late clinical failure	1 (2%)
Adequate Clinical and Parasitological Response at D28	50 (98%)
Adequate Clinical and Parasitological Response at D28 after PCR	51 (100%)

Fever and parasites clearance time

Fever decreased very significantly during treatment period, from day 0 to day 2. On the day of enrollment, axillary temperature run from a minimum of 36.2 ° C (history of fever) to a maximum of 38 ° C (Table 1).

Tolerability and safety

A predominance of loss of appetite (10.3%) followed by cough (7%) abdominal pain and vomiting (3.5%) were reported (Figure 2). No deaths and no cases of severe malaria were seen during the study. All adverse events observed were mild (Figure 2).

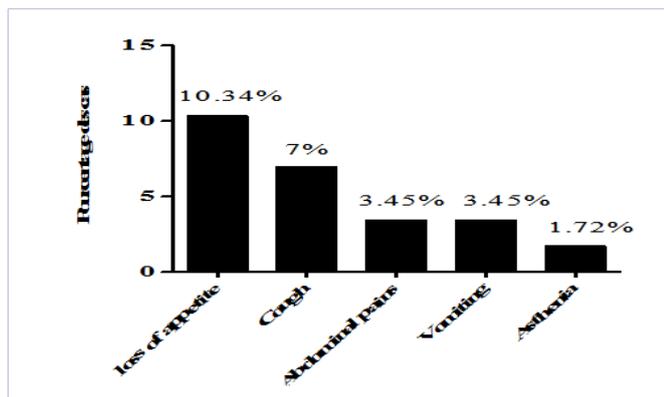


Figure 2: Adverse events

Polymorphism of the K13-propeller gene

The K13-propeller gene was amplified and sequenced in 58 isolates of *P. falciparum*. After alignment with the reference sequence PF3D7_1343700, all were of the wild type, without detectable polymorphism.

Discussion

Malaria remains a major public health problem in developing countries. Prompt access to effective antimalarial treatment such as Artemisinin Based-Combination Therapies (ACT) proves to be an essential tool for controlling the disease. In this study, ASAQ was proved to be effective treatment for uncomplicated falciparum malaria, as evidenced by a PCR-corrected parasitological efficacy of 100%. The present study was conducted to provide supporting evidence for the clinical efficacy of ASAQ, which was adopted and implemented with AL as anti-malarial drug policy in Chad since 2005. The efficacy assessment of ACT has also shown a high efficacy level of ASAQ in neighboring countries of Chad. In Nigeria, a bordering country of Chad, Oguiche et al, found a polymerase chain reaction-corrected parasitologic cure rates on Day 28 of 98.3% with ASAQ [11]. In the same country a ACPR-corrected results on day 28 was 95.8% has been observed with ASAQ during a study conducted by Falade et al, [13]. In Central African Republic, another bordering country, a 28-day therapeutic efficacy study of ASAQ conducted by Djalle et al, in Bangui indicated 93% of ACPR-corrected at day 28 [14]. It seemed that ASAQ is more efficacious in Chad than in Central African Republic potentially due to drug pressure higher in this country. Some studies conducted elsewhere in Africa showed good efficacy of ASAQ [11,15,16,17]. In addition to high cure rates, rapid PCT and FCT have been observed with the drug. Although numerous studies carried out in malaria endemic countries had shown good efficacy and safety of ACT for the treatment of uncomplicated malaria, the conditions of clinical trials do not fully reflect real field situation. Results from studies conducted with unsupervised malaria treatment showed low cure rate after adjustment by day 28 [16,18,19,23]. ASAQ was well-tolerated, similar to many other studies with AEs mostly mild and not linked to the administered treatment [20,17,11]. Safety and tolerability monitoring of ASAQ and other forms of ACT should continue in a standardized manner. Unfortunately pharmacovigilance networks are not

implemented in most settings where ACT is routinely used. This therapeutic efficacy study had several limitations. First this study of drug efficacy have limited follow-up to 28 days, the minimum recommended by WHO, and thus only the short-term effectiveness has been assessed [24]. Any additional recurrences beyond this time frame were not captured (42 days). Secondly drug levels were not tested and challenge of getting reliable safety recall information from children our study population. The results of this study demonstrate that ASAQ remain efficacious treatments for uncomplicated *P. falciparum* malaria in Chad. There is no evidence at this time that a change in regimens is warranted. However, continued monitoring of drug efficacy, following WHO recommendations, is needed. In the bulk of the sub-Saharan African countries, malaria drug policies relay mostly on the use of the artemisinin combination drugs treatment. To avoid the pitfall known with chloroquine and to preserve as long as possible the effectiveness of these ACTs, a better understanding of the underlying mechanisms associated with resistance or loss of susceptibility to these combinations is necessary to ensure an optimal use [14]. Thus, in the present study, the DNA of 58 isolates of *P. falciparum* was analyzed to check mutations associated with resistance to the ACTs. As previously reported and in line with some previous work implemented within sub-Saharan Africa, no parasite of the analyzed sample harbored any mutation neither synonymous nor non-synonymous. This result is in conformity with the clinical data with no records of any therapeutic failure. Our result is similar to that reported in Benin and India where the analysis of *Kelch* 13-propeller sequences indicated that all isolates were of wild type [20-22]. All were no synonymous mutations [25]. The absence of mutation in the gene k13-propeller in our study could explain the sensitivity of the parasite to ASAQ. However, the results of our study could not be inferred to the whole country, even to the Western Mayo-Kebbi region, since only one Pala site hosted the study. The geographical and epidemiologic settings within this Western Mayo-Kebbi region differed from one point to another. Thus, additional studies are needed in order to increase samples size for robust analysis and relevant resistant data from Chad.

Conclusion

Artesunate-Amodiaquine (ASAQ) has been shown to be safe and highly effective in the treatment of uncomplicated *P. falciparum* malaria for children in Pala. No evidence of the emergence of artemisinin resistance in Tchad was found upon investigation of mutations in the k13 propeller domain. However, additional clinical and molecular studies need to be performed in different parts of the country to provide clear and relevant data related to drug resistance in Chad.

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Conflict of interest

The authors state that there is no conflict of interest.

DH and PR are staff members of the World Health Organization. DH and PR alone are responsible for the views expressed in this publication and they do not necessarily represent the decisions, policy or views of the World Health Organization.

Ethics

The procedures followed were in accordance with the ethical standards of Helsinki Declaration. Consent form was signed by parent or legal guardian of children included in the study.

Approval was given by the Ministry of Public Health under ethical clearance N° 2192/PR/PM/MSP/SE/SG/DGAS/DSPELM/DMTNT/PNLP/15 and by WHO ERC.

Abbreviations

ASAQ: Artesunate-Amodiaquine; SP: Sulfadoxine-Pyrimethamine; AL: Artemether-Lumefantrine; TBS: Thick Blood Smear; NPME: National Program For Malaria Eradication In Chad; MPH: Ministry Of Public Health; DS: Sanitary District; SNPs: Single Nucleotide Polymorphisms; IPCI: Institut Pasteur Of Côte d'Ivoire; NCBT: National Center For Blood Transfusion; WHO: World Health Organization; PALAT: Support Project For Malaria Control In Chad; UNDP: United Nations Development Program; GMP: Global Malaria Program; ACT: Artemisinin-Based Combination Therapy; ACPR: Adequate Clinical And Parasitological Response; LPF: Late Parasitological Failure; LCF: Late Clinical Failure; ETF: Early Therapeutic Failure; LPV: Lost Patient View; PRS: Patient Removed From Study; MPD: Mean Parasite Density; DP: Parasite Density; CI: Confidence Index; Ds: Standard Deviation.

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