ORIGINAL PAPER

Status of the artemisinin resistance-associated PfATPase6 S769N mutation in *Plasmodium falciparum* infections of Lusaka Urban District, Zambia.

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ABSTRACT

Objective: Artemisinin derivatives constitute a key component of the present-day treatment for *Plasmodium falciparum* malaria. In Zambia the national malaria policy was revised in 2003 to replace chloroquine mono-treatment with artemisinin combination therapy (ACT). Resistance to artemisinin is associated with a S769N point mutation in the sarcoendoplasmic reticulum calcium-dependant ATPase6 (SERCA-PfATPase6) gene of *P. falciparum*. However, the baseline or current levels of this mutation in Zambia remain unknown.

The present study was aimed at determining the prevalence of the putative artemisinin resistance marker and the extent to which the recommended ACT (artemether-lumefantrine) was in use in Lusaka Urban district.

Design: This was a cross sectional prospective study. Using a nested PCR and allele specific restriction enzyme digestion strategy, *P. falciparum* infections from ten sites in Lusaka urban district were assayed for the prevalence of the PfATPase6 S769N mutation. The availability of current ACT and the extent to which it has been used since introduction were assessed using interview by questionnaire.

Main Outcome: The PfATPase6 S769N mutation was not found on any of the infections analyzed in the present study. Artemether-lumefantrine ACT was readily available in both government-owned health centres and private drug stores as first line malaria treatment in Lusaka urban district.

Conclusion: The absence of the PfATPase6 S769N mutation suggests 100% artemisinin sensitivity unless a different resistance mechanism exists. Continued resistance monitoring and investigation of other potential molecular markers is recommended as wider ACT use is scaled up in the country.

INTRODUCTION

Malaria persists as a leading cause of morbidity and mortality in Zambia, claiming more than 4 million clinical cases and 50,000 deaths annually on the country's population of 10.6 million people [1,2].

In Zambia *Plasmodium falciparum* accounts for approximately 95% of malaria cases, with *P. malaria* contributing 3 % and *P. ovale* 2 %. *P vivax* is rare [3].

In vivo efficacy studies that were conducted in different parts of Zambia between 1995 and 2000 demonstrated levels of CQ resistance ranging from 24% to 52% [4]. In an effort to reduce the malaria disease burden, the Ministry of Health adopted artemisinin combination therapy (ACT) and replaced former mono-therapy options in management of uncomplicated malaria as recommended by WHO[1]. Artemether-lumefantrine,

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therefore serves as the first line drug for the management of uncomplicated malaria except in children below 5kg of weight and pregnant women, where its use is not yet registered. Sulphadoxine/pyrimethamine (SP) became the drug of choice in these excluded groups. During the transition period to ACT adoption sulphadoxine-pyrimethamine (SP) was also used as the first line drug and quinine as the second line drug for malaria treatment [5].

Although artemisinin and its derivatives are the most potent and rapidly acting antimalarials, artemisinin resistance has been reported in murine models of malaria [6]. More recently, *in vivo* and *in vitro* artemisinine resistance has been documented among human falciparum malaria infections in South East Asia [7, 8]. This calls for increased vigilance and a coordinated and rapid deployment of drug combinations to help curtail the escalation and spread of resistance.

Studies of *in-vitro* resistance to artemisinin in field isolates from Senegal, French Guiana and Cambodia point to sarco/endoplasmic reticulum calcium-dependant *Plasmodium falciparum* adenosine triphosphatase6 *(SERCA-PfATPase6)* gene as the target for artemisinins [9]. Resistant isolates, which occurred mostly in areas with uncontrolled use of artemisinin derivatives, carried a *Pf*ATPpase S769N mutation [9].

The main objective of this study was to determine the prevalence of the putative artemisinin resistance marker, *Pf*ATPpase S769N mutation, in Lusaka urban district. Specific objectives were to (i) identify falciparum malaria infections using microscopy and PCR; (ii) genotype falciparum malaria infections on the *Pf*ATPpase amino acid codon 769; and (iii).determine the availability of the recommended ACT (artemether/lumefantrine) and the extent to which this drug regimen had been in use since its introduction, in Lusaka urban district.

MATERIALS AND METHODS

Study area and population

The study was conducted at ten randomly selected urban health centres in residential areas of Lusaka urban district. A malariometric survey showed the district to experience hypoendemic malaria transmission [10]. With no prior data on prevalence of the *Pf*ATPase6 S76N mutant in the area, the study was based on 104 PCR-positive field samples, allowing estimation of the proportion of the mutant with 9.6% precision and 80% study power at 95% confidence level.

Sample collection

Patients were screened for malaria using microscopy of Giemsa-stained thick smears and blood was also collected on Whatman® 3MM filter paper that had been previously serrated for blood sample collection. Air-dried filter papers blotted with blood were individually packed in envelopes, transported and stored at room temperature pending DNA extraction. Sample collection was carried out between September 2006 and April 2007.

Inclusion and exclusion criteria

The study was based on consenting thick film-positive *P. falciparum* of all ages. Only residents defined as having lived in Lusaka urban district for more than three months were included in the study. Questionnaires were only administered to individuals who admitted to having suffered from malaria since the introduction of Coartem®.

Administration of questionnaires

Questionnaires were administered to Lusaka residents visiting the selected health facilities, regardless of the reason they were visiting the health centre. The respondents were a separate group from those who gave blood. The number of respondents at each health centre was proportional to the number of people attended to at each centre in the previous year, 2005. The administrators of the selected health centres and workers/owners of randomly selected drug stores near each health centre and Lusaka town centre were interviewed about Coartem® stocks and sales.

Molecular analyses

Extraction of parasite DNA was carried out using the chelex method [11]. DNA extract for each sample was subjected to nested PCR amplification with primers flanking nucleotide codon 2307of the PfATPase6 gene. Both the primary and secondary reactions comprised 2µL template, 0.25µM primer, 1.5mM $MgCl_2$, 200µM dNTP's, 1X PCR buffer and 1U Tag

DNA polymerase, in 25µL reactions. Amplification cycles for both primary and secondary reactions consisted of an initial denaturation at 94^{0}C for 2 minutes, followed by 25 cycles of denaturation at 94^{0}C for 45 seconds, annealing at 46^{0}C for 45 seconds and extension at 65^{0}C for 1 minute, final extension was run at 65^{0}C for 2 minutes.

Allele-specific Restriction Enzyme Digestion

Internal primers for the nested PCR amplification (2307FW-EN and 2307RV-EN, Table 1) were engineered to create Csp6 I restriction sites. One site, at *Pf*ATPase6 nucleotide codon 1916, serves as the internal control for the restriction digestion assay, which is always cut by the enzyme (Table 1). (Position for table 1)

Tables

PRIMER (nucleotide position)	SEQUENCE	PRODUCT (bp)
2307FW (1908..1926) 2307RV (2393..2368)	5'-TGA GCA TGG CAC AAG TTT T -3' 5'-TCA ATA ATA CCT AAT CCA CCT AAA TA -3'	486bp (primary AMP)
2307FW-EN (1908..1926) 2307RV-EN (2339..2307)	5'-TGA GCA TGG **t**AC AAG TTT T-3' 5'-TCA TCT GTA TTC TTA ATA TTT AAA TCT **g**TA CTA -3'	432bp (secondary AMP)
Csp6I Restriction Enzyme Digestion: Undigested: 432bp Wild type (Ser-2307): 390bp, 32bp, 10bp (enzyme cuts at 1917 on ATFW -EN, and at 2307 on ATRV -EN) Mutated (Asn-2307) : 422bp, 10bp (enzyme cuts at 1917 on ATRV -EN only)		

Table 1 Primers used in PCR and allele-specific restriction enzyme digestion for genotyping PfATPase6 codon 76 (bold shows nucleotide substituted to create Csp6 I restriction site (-GTAC-).

The other site, at nucleotide codon 2312, only occurs if the *Pf*ATPase S769N mutation is present. Csp6 I digestion assay comprised 4µL of secondary PCR amplicon (432bp), 1X new England buffer B, and 1.5U of Csp6 I restriction enzyme, in 30µL reactions. Digestion assays were incubated for 12 hours at 37 ^{0}C. PCR amplicon and restriction digests were analyzed by electrophoresis on a 2% ethidium bromide-stained agarose gel and visualized under UV transillumination. Restriction digests were loaded in 15 µL volumes per lane while undigested product was run in 5 µL loading volumes. Band sizes were measured using Kodak 290 gel imaging analysis software.

Data analysis

Data were analyzed to determine the baseline prevalence and 95% CI of PfATPase S769N mutation in Lusaka urban district.

RESULTS

Use of antimalarials

Of the 119 respondents interviewed in Lusaka urban district 38 (31.9 percent) of the respondents had taken artemether-lumefantrine (Coartem®) since its introduction in Zambia and 83 (69.7 percent) knew someone who had used Coartem for malaria treatment. Only 3 (2.5%) of the respondent had taken chloroquine from the time this drug was withdrawn from government health centres. The rest of the respondents had taken SP and other anti-malarial drugs, 34 (28.6 percent) and 15 (12.6 percent) respectively. Examples of the other antimalarials that the respondents used were Halfan® (Halofantrine), quinine® and Co-Arinate®.

Of the 20 various drug store owners/workers who were interviewed at their drug stores 15 had Coartem® in stock and 5 of these also had chloroquine. Hence, all the drug stores that had chloroquine also stocked Coartem® the remaining 5 neither had chloroquine nor Coartem in stock. Chloroquine stocks were withdrawn and replaced with Coartem® from all the government owned health centres in Lusaka urban district following a workshop for health workers in March 2004 (LUDHMT, Personal communication).

Falciparum malaria infections

Approximately 19,800 patients from 10 Lusaka urban district health centres were screened for malaria between September 2006 and April 2007. Of these 161 (0.8 percent) were positive for malaria by microscopy of Giemsa-stained thick smears.

The Prevalence of the *SERCA-PfATPase6* mutation associated with *P. falciparum* resistance to artemisinin.

Of the 161 malaria positives by microscopy, 104 were subjected to PCR genotyping for estimating prevalence of the *Pf*ATPase codon S769N mutation. The remaining samples did not amplify. All the 104 (100 percent) infections carried the artemisinin sensitive wild type allele, S769 (Figure 1).

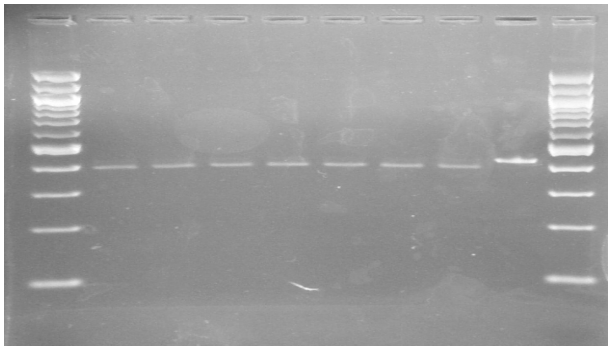

Figure 1- Agarose gel showing Csp6 I digest of *PfATPase6* codon 2307 in parasites from Lusaka urban district. All infections carried the wild type (artemisinin sensitive) allele Ser-2307, appearing as a 390bp band (lanes S1-S6), 1F11 is the laboratory standard carrying the wild type (positive control). Lane 8 shows undigested secondary amplicon of 1F11 (432bp). L, New England Biolabs 100bp ladder.

DISCUSSION

This study showed that the *Pf*ATPase6 S769N mutation associated with resistance to artemisinin had not yet appeared in Lusaka district. The absence of this mutant would seem to suggest full artemisinin sensitivity at the time of the study, unless a different mechanism of resistance occurs in the area.

It was noted that of the initial 161 microscopy positives the number that amplified in PCR assays was 104. Since PCR-based diagnostic methods for malaria surpass microscopic methods with respect to sensitivity and specificity [12], it would be expected that PCR should detect more infections than microscopy. However, because this *P. falciparum*-specific PCR was performed only on microscopy positives, fewer PCR positives can be expected for several reasons, such as microscopy false positives due to species other than *P. falciparum*[13, 14], artefacts etc. On the other hand, PCR false negatives are also possible to a lesser extent, due to variable amounts of blood spotted on filter paper strips, PCR inhibition etc. Be that as it may, this would not cause any bias to the study.

The results showed that 31.9 % of respondents had used Coartem® for malaria treatment since its introduction, which was about 3 years earlier. This may be a reflection of the low background malaria prevalence in the hypoendemic Lusaka district, as evident from the 0.3% parasite rate found during the study and from previous malariometry [10]. Coupled with the policy requirement to administer ACT only on confirmed (RDT or microscopy) positives, it is possible that relatively few people would have actually had true malaria infection during the recall period. Moreover, unlike other antimalarials such as SP (costing K1500 - K3000 per course), over-the-counter access to the less affordable artemether-lumefantrine (K35, 000 - K45, 000 per course in the same pharmacies) was bound be relatively limited, except primarily through health facilities, following a requisite confirmatory test.

According to Jambou *et al* [9], field isolates that exhibited *in vitro* resistance to artemisinin occurred in areas with uncontrolled use of artemisinin derivatives. The observed absence of the artemisinin resistance marker in the current study may be due to relatively regulated conditions under which the drug is being used in Zambia, i.e. as combination regimens only, in addition to being a newly introduced antimalarial for which resistance has not yet developed. Despite the absence of evidence for *P. falciparum* resistance to artemisinin apparent in this study, there is need for continued monitoring to maintain evidence-based decisions by policy markers. Continued regulated use of ACTs as first line treatment for uncomplicated malaria is recommended.

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