academicJournals

Vol. 8(47), pp. 3811-3814, 19 November, 2014
DOI: 10.5897/AJMR2014.6785
Article Number: 5178F4548990
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

The use of Artemisia tea and occurrence of single nucleotide polymorphisms in the *PfATPase6* gene

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Received 18 March, 2014; Accepted 18 November, 2014

The herb *Artemisia annua* L. was used in ancient Chinese medicine to treat malaria and fevers. In a Ugandan community (Wagagai flower farm) located at the shores of Lake Victoria in Entebbe Municipality, tea infused with dried leaves of this herb (Artemisia tea) is drank by employees. The effect of drinking Artemisia on changes (single nucleotide polymorphisms, SNPs) in *Plasmodium falciparum* candidate resistance genes is not known. This study therefore investigated the use of Artemisia tea and the occurrence of SNPs in the PfATPase6 gene. A section of the *PfATPase6* gene of 1940 bps was amplified by polymerase chain reaction (PCR) and the amplicons purified, followed by sequencing. Using CLC MainWorkBench software, the sequences were aligned and with *P. falciparum* 3D7 as reference sequence, SNPs were manually inspected in the sequences (n=17). Single nucleotide polymorphisms were observed at two positions from the 17 sequences: SNP T1707A occurring in 2/17 (11.8%) of the sequences and SNP T2694A in (10/17) 58.8% of them. This study identified two SNPs in the PfATPase6 gene of *P. falciparum* isolates obtained from employees of Wagagai flower farm who were using Artemisia tea to protect themselves against malaria. However, based on studies done elsewhere, these SNPs where not associated with artemisinin resistance.

Key words: Artemisia tea, *Pf*ATPase6 gene, single nucleotide polymorphisms.

INTRODUCTION

Artemisia annua L. has been used in herbal treatment of intermittent fevers and malaria by the Chinese for centuries; the active ingredient, artemisinin was isolated from this herb in 1971 (Hsu, 2006). Artemisinin based combination therapies (ACTs) was recommended and is currently used in the treatment of uncomplicated malaria (WHO: Guidelines for treatment of malaria, 2010). However, because ACTs are relatively expensive and may not be readily available in many areas with malaria in Africa, herbal preparations of *A. annua* L. is currently being promoted to treat malaria (Willcox et al., 2011). In a Ugandan community, tea infused with dried leaves of *A.*

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annua L. (Artemisia tea) is drank to cure or protect against malaria and it has been shown to be efficacious (Ogwang et al., 2011). However, the quantity of artemisinin in the tea is low; plants grown in East Africa yield low quantities of artemisinin ranging from 0.2 to 0.35% (Wilcox et al., 20l1), as compared to those grown professionally in Europe with a quantity of between 0.5 and 0.70% of artemisinin (Mueller et al., 2000). Artemisia tea prepared with 5 g of dried leaves contains about 60-70 mg of artemisinin and if administered for 5 days, reaches 320 mg, this is in contrast to the usual clinical dose of the artemisinin derivatives (artesunate or artemether) which is between 500 and 1000 mg total within 2 - 6 days of treatment (de Vries and Dien, 1996).

There is therefore concern that the low quantity of artemisinin in the preparation may not be sufficient to clear all *P. falciparum*, leaving behind parasites which may develop resistance to artemisinin (WHO: Position statement on the effectiveness of Non-Pharmaceutical Forms of *A. annua* L. against malaria, 2012). Resistance to antimalarials is associated with SNPs in *P. falciparum* genes; *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) to Chloroquine resistance (David et al., 2000; Wallis et al., 2004), Dihydropteroate synthetase (dhps) and dihydrofolate reductase (dhfr) genes to sulphadoxine-pyrimethamine (Pedro et al., 2005). Earlier studies suggested PfAPase6 gene to modulate artemisinin resistance (Uhlemann et al., 2005; Jambou et al., 2005), however currently polymorphisms in the Kelch and cysteine protease falcipain-2 genes have also been implied (Ariey et al., 2014). This study being reported was done in 2010, at that time the roles of Kelch and cysteine protease falcipain-2 genes to artemisinin resistance had not yet been identified. The main objective of this study was to determine whether the use of Artemisia tea is associated with Single nucleotide polymorphisms which may occur in the PfATPase6 gene in *P. falciparum.*

MATERIALS AND METHODS

Sample collection

The study was done in Wagagai flower farm, located at the shores of Lake Victoria in Entebbe. *P. falciparum* isolates were from adults, who participated in Artemisia tea study performed in 2010; samples were collected with consent from farm management and workers and also the study received ethical clearance from the Higher Degree Committee of Makerere University (Ogwang et al., 2011). Participants were all employees of the farm; here Artemisia tea was administered at least once a week by farm management, as a preventive measure against malaria. This had been going on for a period of about four years. Samples were collected from a health centre which belongs to the farm and is located within its premises. Thick Giemsa-stained blood smears and blood spots on 3 mm Whatman 903® FTA card were obtained from those who showed fever and related symptoms of malaria. All patients positive for malaria were treated with Artemether-lumefantrine. FTA cards (n=46) from malaria positive patients by microscopic examination were randomly selected from those archived for genotyping. Another 12 FTA cards from malaria positive patients by microscopy examination were obtained from a clinic in Entebbe town from patients who were not exposed to Artemisia tea.

Plasmodium falciparum speciation

DNA was extracted from blood spots on 58 FTA cards (n = 46 from Wagagai, n = 12 from the clinic) by chelex (Sigma-Aldrich), in 100 µL of sterile water as described by Dokomajilar et al. (2007). A nested PCR was used to amplify about 200 bp of the 18S small subunit ribosomal DNA. Primers used were rPLUf: 5'- CCT GTT GTT GCC TTA AAC TTC 3'/ rPLUf: 5'- TTA AAA TTG TTG CAG TTA AAA CG 3' for the primary reaction and rFLUf: 5'- TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT 3'/ rFLUr: 5'- ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC 3' (Dokomajilar et al., 2007), for the nested reaction. The amplifications were performed in 20 µl reaction containing, 0.5 µM of forward and reverse primers, 200 µM dNTPS, 1x Phusion HF Buffer containing 1.5 mM $MgCl_2$, 0.02 U of Phusion™ DNA polymerase (FINNZYMES Oy), 3 µl DNA template and 12.2 µl PCR grade sterile water for the primary PCR reaction; 1 µl of amplicons from the primary reaction and 14.2 µl PCR grade sterile water for the nested reaction. Genomic DNA from *P. falciparum* 3D7 was used as a positive control and PCR grade water as negative control. Thermocycler (Techno® TC-412) was used and the conditions were: 98°C for 1 min, 35 cycles of 98°C for 10 s, 64°C for 30 s for primary reaction, for nested reaction 68°C for 30 s annealing, 72°C for 30 s, and 72°C for 5 min for all reactions. The products were electrophoresed on 2% agarose gel stained in ethidium bromide with 1xTBE (Sigma Aldrich) as the running buffer and visualized using uvitec illuminator then documented.

PfATPase6 gene analysis

Amplification of *PfATPase6* gene was performed on only those samples confirmed to be positive for *P. falciparum* by the *P. falciparum* specific PCR (n=35 from Wagagai, n=7 from the clinic). A section of the of *PfATPase6* gene containing bases 1138 to 3078 (1940 bps), where SNPs were reported to occur (Ferreira et al., 2008; Maman et al., 2009) was amplified by PCR. The primers used for this PCR reaction were 5F: 5'- ACCGTGTTTCATTTGTTTAGAG 3'/10R: 5'-TGTGCTGGTAATCCGTCAG 3' (Ferreira et al.2007). The amplification was done in 25 µl PCR reaction mixture containing, 0.1 µM of forward and reverse primers, 200 µM dNTPS, 1xPhusion HF Buffer containing 1.5 mM $MgCl_2$, 0.04 U of Phusion™ DNA polymerase (FINNZYMES Oy) and 3 µl of DNA template. The PCR conditions were:9 8°C for 1 min, 35 cycles of 98°C for 10 s, 62°C for 30 s, 72°C for 90 s and 72°C for 10 min. The amplicons were electrophorised on 1% agarose gel stained with ethidium bromide in 1xTBE running buffer and visualized using uvitec illuminator. Amplicons of the correct size (1940) were cut out using a sterile scalpel and DNA extracted and purified using GENJET™ Gel Extraction Kit (Fermentas). A total of 25 purified amplicons (n=16 from wagagai, n=9 from the clinic) were selected and sent to Macrogen Korea (http://dna.macrogen.com) for sequencing. The sequences were aligned with *P. falciparum* 3D7 sequence obtained from Plasmodium data base with accession number of PFAO310C, using the CLC MainWorkBench software version 6.0.2 (http://www.clcbio.com) and SNPs manually scored.

RESULTS

After editing the sequences for any ambiguity, 17 out of the 25 were consistent, and only these 17 sequences were inspected for SNPs. Two SNPs were observed (Table 1).

Table 1. A PfAPase6 gene substitution from *P. falciparum* isolates from Entebbe.

Nucleotide position	Normal allele	Mutant allele	Mutation type	Normal amino acid	Mutant amino acid	n/N
1707	T	A	Synonymous	N	K	2/17
2694	T	A	Non-Synonymous	I	I	10/17

n = number of samples with mutant allele; N = total number of sampled sequenced and inspected for SNPs.

A synonymous polymorphism at position 2694 where a base Thiamine (T) was substitution with Adenine (A) was observed in 10 isolates (n = 7 from Wagagai isolates, n = 3 from the clinic isolates). Also, a non synonymous polymorphism involving base change from T to A at position 1707 was observed in 2 isolates all from Wagagai samples. This synonymous polymorphism leads to amino acid change from Asperagine (N) to lysine (K) at position 569. Most of the isolates had only one polymorphism; however one isolate had both T2694A and T1707A polymorphisms.

DISCUSSION

The consumption of low doses of artemisinin, in Artemisia tea for a prolonged time may influence resistance to artemisinin its self (World Health Global Malarial Programme: Position statement on the effectiveness of Non-Pharmaceutical Forms of *A. annua* L. against malaria, 2012). Information on Artemesia tea use and its possible association to mutations in artemisinin resistance candidate genes is scarce. This study performed in 2010, genotyped SNPs in the PfATPase6 gene in *P. falciparum* from patients in a Ugandan community who were using Artemisia tea in management of malaria. The two SNPs observed in this study, were also reported by other studies done elsewhere; T1707A in *P. falciparum* from Niger, Zanzibar and Tanzanian (Maman et al., 2009; Dahlstrom et al., 2008). Single nucleotide polymorphism T2694A was the most prevalent. This SNP was also observed in *P.falciparum* isolates from South American (Ferreira et al., 2007; Brasil et al., 2012), and those of the Greater MeKong Subregion (Miao et al., 2013) and was also the most prevalent in the regions. It seems that the two alleles (A or T) are fairly distributed in *P. falciparum* populations (Ferreira et al., 2007), this distribution may not be a result of artemisinin pressure but possibly due to parasite natural evolution. An *in vitro* study of the *P. falciparum* response to artemether or artesunate revealed no association of this mutation with reduced sensitivity to these drugs (Ferreira et al., 2007). Also, Maman et al. (2009), observed no association between T1707A SNP and parasitemia levels in individuals with *P. falciparum* malaria. It is therefore unlikely that these mutations could have been selected by Artemisia tea use or even artemisinin pressure. The T2306A SNP responsible for amino acid substitution from S to N at codon 769 previously reported by Jambou et al. (2005) as a putative marker for artemisinin resistance was not observed in this present study. Current available data indicate that the PfATPase6 gene may not be the one under selection in the case of artemisinin resistance (Miao et al., 2013; Tanabe et al., 2011). Recent studies have identified SNPs in the Kelch and cysteine protease falcipain-2 genes (Ariey et al., 2014) as potential markers for artemisinin resistance.

In conclusion, this study observed two SNPs, however based on other studies done elsewhere, these SNPs where found not to be associated with artemisinin resistance. ACTs still continue to remain efficacious in Uganda (Muhindo et al., 2014), but constant monitoring of practices which may predispose selection of artemisinin resistance *P. falciparum* is imperative. Especially, artemisinin resistance was confirmed in some parts of Asia (Phyo et al., 2012). Future molecular surveillance studies should be focused on Ketlch and cysteine protease falcipain-2 genes as a study by Ariey et al. (2014), associated mutations in these genes, with decreased *in vitro* artemisinin sensitivity and delayed parasite clearance in patients after treatment with artemisinin in Asia.

Some of the limitations of this study were: the few number of isolates studied. The smaller sample size may not have been representative enough for Wagagai community. Also, we did not perform PCR to distinguish between single and multiclonal infections, therefore it was not possible to infer on the clonal diversity of the isolates.

Conflict of Interest

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank Dr Patrick Ogwang for allowing them to use samples he collected in 2010. They are also grateful to Prof. Enock Matovu of Makerere University College of Veterinary Medicine and Biosecurity for allowing them to use his laboratory and reagents to do this work.

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