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SEQUENCE ANALYSIS OF K13 PROPELLER GENE POLYMORPHISM OF *PLASMODIUM FALCIPARUM*-INFECTED PATIENTS IN EGYPT

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Abstract

Artemisinins (ART) are world's most effective anti-malarial drugs, which replaced chloroquine and other antimalarials after emergence of resisting strains to them. They caused a marked malaria deaths' reduction with a hope of future world free of malaria. Unfortunately, Artemisinin-resisting *Plasmodium* strains have emerged in many Asian Countries, although rarely detected in Africa till now; regular screening of resistance genes is a must as Africa recorded the highest endemic and pandemic cases. So, emergence of ART resistance would be risky due to the limited treatment facilities. This study screened K13-propeller gene polymerphisms related to ART resistance in *P. falciparum*-infected patients at Abassia Fever Hospital, Egypt. Clinical and laboratory evaluations of patients were performed; those who displayed delayed parasite clearance were screened for polymorphisms of the K13-propeller gene by nested PCR and sequencing. Sequences were retrieved from 20 (90.9%) of the 22 samples with delayed clearance. Seven K13 gene mutations were discovered in 9 samples (9/20) (i.e., 45 %) of all tested sequences. Two of the seven-point mutations were synonymous, whereas the other five were non-synonymous (R410I, N772K, S775F, C778M, H783S) but not identified as a cause of delayed clearance.

Key words: Egypt, Patients, Malignant malaria, Artemisin, K13-propeller gene polymerphisms

Introduction

Malaria is one of the most common parasitic infections in the world that infect more than 200 million cases. Africa accounts for an excessively high proportion worldwide (WHO, 2019). Africa shared with 94% of global malaria cases and deaths that exceeded 400,000 deaths, most of them were children (WHO, 2021). Global mortality rate ranged from 0.3 to 11-30% in severe cases of tropical regions (Yang *et al,* 2017; Talapko *et al,* 2019). Although malaria is interrupted in Egypt, the increasing number of imported cases is a risk to re-emergence especially with the presence of the vectors, *Anopheles pharoensis* and *A. sergenti* (Tabbabi *et al,* 2020), introduction of new vectors from south Sudan via Toshka (Shoukry and Morsy, 2011), and shared in Multination Peace Forces (El-Bahnasawy *et al,* 2014)

Efficacy of antimalarial drugs is a powerful guarantee for the control and elimination of malaria. Thus, continuous emergence of drug-resistant strains is a threatening agent of emerging disaster (Trape, 2001). For example, the emergence of resistance to chloroquine in the past two decades was responsible for the 2-3 folds increase in number of malarial deaths (Ursos and Roepe, 2002).

Artemisinin proved a great therapeutic efficacy that made it a good substitute to chloroquine especially after spread of chloroquine-resistant strains. Artemisinin-based combined therapy (ACT) was recommended as the first-choice for uncomplicated *P. falciparum* infection (WHO, 2006). Its use in endemic areas was associated with a marked reduction of morbidity and mortality of malaria in the first 10 years of its use (Ashley *et al,* 2018). The WHO reported that the absolute reduction in malaria deaths in the African region was the largest, from 533,000 in 2010 to 380,000 in 2018. Artemisinins high efficacy in treatment and deaths' reduction encouraged development of the goal of a malaria-free world (WHO, 2015). The WHO

-certified countries that achieved zero original cases increased from 17 countries in 2010 to 25 countries in 2017 and 27 countries in 2018 (WHO, 2019).

However, the ACT curative efficacy started to decline gradually as time passed. The first *P. falciparum* resistance was in 2008 in Western Cambodia, which spread to other nearby South Asian Countries (Duru *et al,* 2016). The ACT resistance became a major threat to the global malaria elimination campaign (Mok *et al,* 2021).

The ACT resistance cases were in the form of delayed parasite clearance that was mainly mediated by mutations in the kelch propeller domain K13-propeller, a gene responsible for parasite survival within host's RBCs (Ariey *et al,* 2014). The K13 mutations led to a malfunctioning developmental program of *Plasmodium* inside the RBCs e.g., changes of the cell-cycle periodicity, response of the unfolded protein, protein degradation, vesicular trafficking, and mitochondrial metabolism (Mok *et al,* 2021). Association between K13 mutations and artemisinin resistance were mainly reported in Asia, and rare cases in Africa (Yang *et al,* 2017). But, regular screening of mutations was mandatory due to the high African incidence of *Plasmodium* infections, in particular *P. falciparum* artemisinin-resistant cases (Saleh *et al,* 2019).

This study aimed to evaluate the status of the ART resistance-related polymorphisms of the K13-propeller gene in *P. falciparum*-isolates in Egypt.

Subjects and Methods

Ethical considerations: The Research Ethics Committee of the Faculty of Medicine, Menoufia University approved this study. The study's goal was explained to all participants, and they gave informed consents.

Study design: The study was performed on 104 *P. falciparum*-infected patients admitted to Abassia Fever Hospital, Cairo, Egypt. The study extended from November 2016 to October 2017. All patients had positive rapid diagnostic tests and blood films having more than 2 asexual stages of *P. falciparum.* Exclusion criteria were the usage of any antimalarial drugs a week before or recently travel to Africa. All patients went through a full history taking, clinical and laboratory examinations. According to WHO protocol patients were classified into uncomplicated and complicated ones (Gomes *et al,* 2011). Patients were considered complicated if they had one or more of the following criteria: a- cerebral malaria (Coma not ascribed to other causes, Glasgow ≤ 9), b- severe anemia (Hb <7g/dl or hematocrit <20% with parasitemia >10,000/µl), c- acute renal failure (urine output <400mL/24hrs in adults & <12 mL/kg/24hrs in children, and serum creatinine > 3mg/dL, d- pulmonary edema (radiographic changes and severe hypoxemia), e- severe hypoglycemia (Blood glucose <40 mg/dL), f- shock (Systolic blood pressure < 70mm Hg in patients >5 years of age [< 50mmHg in children]), g- abnormal bleeding and/or disseminated intravascular coagulation (spontaneous nasal or gastrointestinal tract bleeding or laboratory evidence of disseminated intravascular coagulation), h- repeated generalized seizures (≥3 episodes within 24hrs), i- metabolic acidosis (arterial pH <7.25 or HCO3 <15mmol/L). j- hemoglobinuria (no secondary to glucose-6-phosphate deficiency hemolysis), k- consciousness status impairment (altered consciousness level), i- prostration or weakness, m- hyperparasitemia (>5% erythrocytes infected or >250,000 parasites/µl in non-immune subjects), n- hyperpyrexia (body temperature > 40ºC), & o- hyper-bilirubinemia (total bilirubin >2.5mg/dL). The patients were divided among two groups, GI: uncomplicated malaria. GII: complicated or severe malaria.

Blood films for parasitic density: Capillary blood from each patient was used to prepare thick and thin blood smears. Smears were air-dried before being stained with 10% fresh Giemsa for 15 minutes and examined microscopically to detect the malaria species and parasite density. Parasites were counted in the thick blood films against white blood

cells (WBCs) assuming a leukocyte count of 8000/µl of blood, if the parasites were more than 100/ field of 100x oil immersion, a thin blood film-calculated parasite density was performed (WHO, 2016).

Follow up of patients for parasite clearance time (PCT): Asexual and sexual parasites were regularly counted on Giemsa-stained thick and thin blood films every 8hrs started immediately before the first treatment dose until three consecutive films were negative for asexual parasites, and blood films were followed-up on the days 2, 3, 7, 14, 21, 28, & 42.

Extraction of parasite DNA and sequencing of *P. falciparum* K13-propeller gene: Blood samples were obtained from all patients and preserved at -20^{o}C. Nested PCR and sequencing were done for patients' samples that showed delayed parasite clearance. The QIA amp DNA Blood Mini Kit (Catalog No. 51104, Qiagen, Germany) was used to extract genomic DNA from 100µL of each who-le blood sample after the manufacturer's instructions. A nested PCR amplification technique was employed to amplify the *P. falciparum* K13-propeller (Ariey *et al*, 2014). Lists of oligonucleotide primers & cycling conditions given (Tab. 1). Total 25µl amplification reaction mixtures contained 8.5-9.5µl of dH2O, 1.0µl of each primer, & 12.5µl of TaqPCR Master Mix (Catalog No. WF10203001, Cosmo red PCR Master Mix (2X), Willow- fort, UK). With the addition of 2.0µl of template genomic DNA produced from the blood samples, primary amplification processes were started. Template for nested PCR was 1µl of primary PCR output. On the 2% Agarose gel, amplified PCR products were added, and PCR products sizes were visually quantified by a 100bp DNA ladder (Catalog No. SM0331, Thermo Scientific GeneRuler DNA Ladder Mix, US).

Nested PCR of K13-propeller products were directly sequenced in both directions, using ABI PRISM® 310 Genetic Analyzer (Thermo Fisher Scientific, USA). K13-propeller nucleotide and amino acid sequences were compared to wild-type amino acids sequence (Gen-Bank accession number, XM_ 001350122) using Clustal W of Bio-Edit 7.0 &MEGA 4.0 programs.

Statistical analysis: Data entry, coding, & analysis were conducted by IBM personal computer with statistical package SPSS version 22 (Armonk, NY: IBM Corp, 2013). Two statistics types were used, descriptive statistics: percentage (%), mean (x), & standard deviation (SD). Analytic statistics: Chi-square test ($\chi 2$) studied association between two qualitative variables. Students'-test of significance compared between two groups with quantitative variables. Mann-Whitney test of significance compared between two groups without normally distributed quantitative variables. A P= < 0.05 was significant.

Results

The socio-demographic analysis showed a higher numbers of males in complicated and uncomplicated patients, but without significant difference between male incidences in both groups. Both groups were cross-matched with one another as to ages. As regards occupations, all complicated ones (100%) worked in outdoor environmental exposure to infection compared to only 39% of complicated ones with a significant difference ($p<0.001$). The comorbidities incidence and/or other risk factors of severity were significantly higher in complicated patients than the uncomplicated ones as to diabetes, obesity, and non-O blood groups.

Only 22(22.88%) of 104 patients showed clinical severity, as hyper-parasitemia, cerebral malaria, metabolic acidosis, acute renal failure, repeated generalized seizures, severe anemia, hyperbilirubinemia, and/or pulmonary edema. Complicated patients showed a significant earlier clinical manifestation on arrival from endemic areas compared to the uncomplicated ones.

Blood films of complicated participants showed significantly higher parasitemia, gametocyte % & density compared to uncomplicated ones. Mean Hb concentration was lower in complicated patients with sig-

nificant difference between both groups. Laboratory follow-up showed that all PCTs were significantly long in complicated patients than the uncomplicated ones. Parasitemia persisted in 95.5% of patients to 42nd day follow-up. It was correlated clinically to rapid recovery of uncomplicated patients that occurred within 3 days post-oral artemether and lumefantrine therapy, and delayed clinical recovery of complicated ones up to 13 days after receiving IV artesunate, & death of 2 patients. Delayed clearance was recorded in 22 complicated patients,

with sequences retrieved from 20 (90.9%) samples. The remaining two samples didn't analyze due to low quality or inadequate quantities of DNA. Of 20 sequenced samples, 7-point mutations of K13 propeller gene were detected in 9 samples (45%). Two of them were synonymous, and five were non-synonymous mutations (R410I, N772K, S775F, C778M, H783S). Mutated sequences were in samples from North Sudan (5 samples), Guinea (2), and Nigeria (2).

The details were given in tables (1, 2, 3, 4, 5, 6, & 7).

Table 1: Primer sequences and cycling conditions used to amplify K13-propeller gene of *Plasmodium falciparum* isolates

primers	PCR	Sequence	PCR cycling conditions	Product size
K13-1	1ry	5′-cggagtgaccaaaatctggga-3′	95 °C 5 min/[95 °C 30 s, 60 °C 90 s, 72 °C90 s] ×	2096
K13-4		5′-gggaatctggtggtaacagc-3′	40 cycles, 72 °C 10 m	
K13-2	Nested	5′-gccaagctgccattcatttg-3′	95 °C 5 min/[95 °C 30 s, 60 °C 90 s, 72 °C90 s] ×	848
K13-3		5′-gccttgttgaaagaagcaga-3′	40 cycles, 72 °C 10 min	

Table 2: Comparison between groups regarding the sociodemographic criteria of participants.

Variables	Uncomplicated (n=82)		Complicated (n=22)		Total (n=104)		χ^2	p- value
	No	%	No	%	No	%		
Male	66	80.5	16	72.7	82	78.8		
Female	16	19.5	6	27.3	22	21.2	0.63	0.429
Age ≤17(years)	9	11.0	2	9.1	11	10.6		
>17	73	89.0	20	90.9	93	89.4	0.07	0.799
Mean ± SD	31.78±12.27		29.86±13.92					
Range	5-62		6-62					
Median	31		27.5					
Occupational								
-Environment (outdoor)	32	39.0	22	100.0	54	51.9	25.84	<0.001
-Environment (indoor)	50	61.0	0	0	60	48.1		
Nationality: Egyptian travelers	25	30.5	5	22.7	30	28.8		
: Non Egyptians	57	69.5	17	77.3	74	71.2	0.51	0.476
Egyptian travelers visited					N0=30			
-North Sudan	12	14.5	3	13.7	15	50.0		
-Nigeria	10	12.2	1	4.5	11	36.7	0.77	0.679
-Guinea	3	3.7	1	4.5	4	60.3		
Foreigners' nationality					N0=74			
-North Sudan	25	30.5	6	27.3	31	41.9		
-South Sudan	10	12.2	4	18.2	14	18.9	0.88	0.928
-Nigeria	10	12.2	4	18.2	14	18.9		
-Chad	9	11.0	2	9.1	11	14.9		
-Guinea	3	3.7	1	4.5	4	5.4		

Table 3: Comparison between groups regarding risk factors of severity.

Items		Uncomplicated group		Complicated group		χ^2	p- value
		NO	%	NO	%		
Pregnancy		9	11.0	4	18.2	0.8	0.364
Diabetes mellitus		4	4.9	11	50.0	28.61	<0.001
Obesity		29	35.4	13	59.1	4.06	0.044
Anemia		69	84.1	20	90.9	0.64	0.423
ABO grouping	A	1	1.2	18	81.8	8.28	0.041
	B	1	1.2	1	4.5		
	O	60	73.2	1	4.5		
	AB	20	24.4	2	9.1		
Hypoglycemia		9	11.0	6	27.3	3.73	0.053
Liver function abnormalities		39	47.6	15	68.2	2.96	0.086
Renal function abnormalities		19	23.2	8	36.4	1.57	0.210

Table 4: Criteriaof severity among complicated patients.

Variables	No	%
High parasitological index >5%	22	100
Glasgow ≤ 9	12	54.5
Multiple convulsions	5	22.7
Metabolic acidosis	11	50.0
Serum bilirubin >2.5 mg/dL	15	68.2
Renal failure	9	40.0
Pulmonary edema	4	18.2

Table 5: Comparison between groups regarding progress of clinical pictures.

Variables	Uncomplicated Group		Complicated group		Test	p- value
	Mean	SD	Mean	SD		
Onset of symptoms (days)	8.93	0.83	6.86	0.80	t=10.48	<0.001
	8.00-9.50	IQR 9.00	6.38-7.50	IQR 7.00		
Duration of recovery (days)	3.31	0.84	13.62	2.71	t=29.52	<0.001
Deaths	No.	%	No.	%	$\chi^2 = 7.60$	0.006
	0	0	2	9.1		

Table 6: Comparison between groups regarding blood results.

Variables	Uncomplicated group	Complicated group	Test of significance	p- value
Pf. parasitemia/µL M±SD Median IQR	30.41±1.38 30.50 28.95-32.00	281818.70±392352.29 200000.45 100000.58-300000.00	Mann-Whitney test= 7.21	<0.001
Pf. gametocyte carriers: [no (%)]	22 (26.8)	11 (50.0)	χ^2=4.30	0.038
Pf. gametocyte density/µL M±SD Median IQR	30.04±1.70 30.00 28.00-32.00	50.25±1.47 50.00 48.88-52.00	t=50.80	<0.001
Hemoglobin (gm/dl) M±SD Median IQR	10.10±1.48 10.00 8.88-11.63	7.70±1.52 7.75 6.50-8.50	t=6.70	<0.001
Anemia (< 10 g/dL): [no (%)]	40 (48.8)	22 (100.0)	χ^2=18.90	<0.001

Table 7: Comparison between groups regarding follow-up by PCTs.

Variables	Uncomplicated group	Complicated group	t-test	p- value
Parasite clearance time (PCT), hours				
PCT median (IQR)	31.88±1.08 32 (31-33)	41.52±2.16 42 (41-43)	29.24	<0.001
PC50 median (IQR)	9.22±0.85 9 (8.5-10)	14.75±1.70 15 (14-16)	21.24	<0.001
PC90 median (IQR)	14.14±0.85 14 (13.4-15)	19.84±1.56 20 (19-21)	22.88	<0.001
PC95 median (IQR)	16.18±0.85 16(15.5-17)	25.89±2.15 26 (25.5-27.0)	32.63	<0.001
PC99 median (IQR)	23.01±0.97 23 (22.4-24)	35.8±3.01 36.5(35.4-37.5)	32.95	<0.001
Fever clearance time (FCT): median (IQR)	6.14±0.85 6 (5.4-7)	70.56±1.11 70.5(69.9-72)	295.13	<0.001
Treatment outcomes	No (%)	No (%)	χ^2	p- value
LPF (late parasitological failure (PCR corrected) n	0	22 (100%)	104.00	<0.001
At day 28, n (%)	0	18/20 (90%)	98.08	<0.001
At day 42, n (%)	0	18/20 (90%)	98.08	<0.001

Discussion

To the knowledge spread of ART-resistant mutants was still limited in Africa. Although it gave a transient relief, regular screening of malaria is mandatory as Africa already shares with more than 90 % of world's malaria cases (Morsy *et al,* 2019; WHO, 2019). The spread of resistance in this region means thousands of deaths especially with poor health care facilities in many African countries (Al-Agroudi *et al,* 2017). Although malaria is interrupted in Egypt, performing such studies on travelers from many malaria-endemic regions provided an opportunity to study the resistance in different African countries who underwent the same treatment protocol at the same time. ART *P. falcipaum* resistance was responsible for 99.7% of Af-

rican malaria cases with commonest drug resistance species (WHO, 2021).

In the present study, higher male prevalence might be as asymptomatic female infections are rapidly cleared than asymptomatic infections of males that usually proceed to cause symptoms (Sumner *et al,* 2021). The 100 % outdoor occupation recorded in the complicated group can be explained by higher rate of exposure to infected mosquitos that led to higher parasitemia and more severe malaria manifestations (Bødker *et al,* 2006).

In the present study, all Egyptian patients gave a history of traveling to Africa in the last 2 weeks, and showed manifestations a week after arrival, which excluded indigenous malaria. This agreed with Al-Agroudi *et al.* (2018) and Abu-Sarea *et al.* (2020) who reported that all malaria patients of the fever hospitals have traveled to one of the endemic African Countries. Most of the patients were Sudanese as reported (Dahesh and Mostafa, 2015) because their rate of travelling to Egypt is higher than other African countries (https://www.unhcr.org/eg/about-us/ refugee-context-in-egypt,accessed 16-3-2021). Also, the incidence and prevalence of malaria in Sudanese population are very high. Annual incidence of confirmed cases exceeded one million (WHO, 2019).

Because ART mainly affects the ring stage of the *Plasmodium,* its malfunction appears clinically as delayed parasite clearance from the blood. A condition was defined as ART-resistance (Pukrittayakamee *et al,* 1994; Gnädig *et al,* 2020). So, the participants who displayed delayed parasite clearance (i.e., suspected ART resistance) were screened for k13 mutations. The use of artemisinin resistance markers has allowed for a more precise characterization of artemisinin resistance; The focus on the K13 mutations because this gene was defined as the dominant causal determinant of the ART resistance in many genetic and clinical data. Some mutations in the k13 were linked to delayed parasite clearance in vitro and in vivo (Ariey *et al,* 2014; Mok *et al,* 2021). But, the protocol of the WHO didn't recognize all non-synonymous K13 mutants as a cause of delayed parasite clearance. A K13 mutation was labeled an associated marker if it had only been proved to associate with delayed parasite clearance in clinical trials and needs to be validated by in-vitro data (WHO, 2018).

In the present study, none of the detected mutations were identified as ART-resistance associated. The small number of detected mutants in our study made it difficult to relate them clinically to delayed clearance especially with the presence of other factors and comorbidities that can delay parasite clearance. The absence of significant mutations, associated with ART resistance in the North and South Sudan agreed with Abdel Hamid *et al.* (2019); Bakhiet *et al.* (2019); Hussien *et al.* (2020), and Mohamed *et al.* (2020), who didn't detect any significant k13 mutations in uncomplicated *P. falciparum* Sudanese patients. Also, Nigeria patients were all free of significant K13 mutations. This can give some relief as Nigeria is the highest African country regarding the prevalence of malaria accounting for > 25% of malaria cases in Africa (WHO, 2019).

The present results agreed with Oboh *et al*. (2018); Igbasi *et al*. (2019), and Abubakar *et al.* (2020) who didn't find significant ART-resistance-associated mutations. Similar to the present results, Ménard *et al.* (2016) reported that Chad and Ghana were free of significant mutations of K13. The presence of delayed clearance despite the nonsignificant ART-resistance-associated mutations may be due to other factors than genotypic mutations (Miraclin *et al,* 2019; Nima *et al,* 2021). In the present study, there were some associating comorbidities e.g., obesity and diabetes which increased the parasite density that delayed clearance detected that agreed with Sowunmi *et al.* (2010). Malaria parasite and its pathogenesis are affected by the metabolic changes that occur with diabetes and obesity e.g., glucose levels markedly affect *Plasmodium* growth (El-Tawdy *et al,* 2018).

Moreover, lipoproteins are vital for parasite cell membranes and endothelial adherence of infected erythrocytes. Diabetes explained asymptomatic parasitemia that persisted in many patients even after hospital discharge and disappearance of symptoms (Wyss *et al,* 2017). Most of complicated patients had one of the non-O blood groups. The O patients were prot-ected from developing severe manifestations by reduced incidence of rosetting. Rosetting proved important in severe malaria pathogenesis by blocking micro-vascular blood flow (Rowe *et al,* 2007). Non-O blood grou-ps are more liable to increase parasite density and subsequently delayed clearance.

Conclusion

The significant ART-resistance-associated mutations were absent in all African patients' samples. Thus, the recorded delayed parasite clearance was not caused by K13 mutations. Complicated patients had one or more risk factors that increase parasite density and led to detected delayed clearance.

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