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EDITORIAL

POLYMORPHISMS OF PFCRT AND PFMDR-1 GENES AND CHLOROQUINE RESISTANCE OF *P. FALCIPARUM* IN WAD MEDANI (CENTRAL SUDAN)

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ABSTRACT

Introduction: Malaria parasite resistant to Chloroquine poses severe and increasing health problems in tropical countries. Monitoring the drug resistance by implementing the molecular markers may be essential to overcome the problem, therefore this study aims to assess the Chloroquine resistance of Plasmodium Falciparum parasite in central Sudan, using molecular markers.
Methods: One hundred and seventy six patients were confirmed P. falciparaum positive. Sixty-four were selected and only forty patients completed the follow-up. In vivo sensitivity assay was used accompanied with standard regimen of Chloroquine phosphate. DNA was extracted from blood on filter paper (day 0) and was used to amplify two genes P. Falciparum transporters gene Pfcrt and multi-drug resistant gene-1 Pfmdr-1.
Results: Among forty patients, 54% responded to Chloroquine regimen with adequate clinical response (ACR), however, 46% showed treatment failure. All treatment failures were treated with Artemether or Quinine. The amplification of Pfcrt gene (n, 18) and Pfmdr 1 gene (n, 29), had shown that 72% of Pfcrt T76 were mutant allele, 22% were K76 wild-type, however, only 5% were mixed alleles T/K. while Pfmdr 1 gene (n, 29) revealed that 55% were wild genotype N 86, 38% were mutant Y 86, and 7% were mixed alleles Y/ N 86.
Conclusion: The high frequency of the mutant Pfcrt 76T gene among P.

Falciparum isolates was consistent with in vivo study supports the hypothesis that Pfcrt 76T gene could be used as predictive marker for Chloroquine susceptibility in epidemiological surveys.

Key words: *P. Falciparum*, drug resistance, *in vivo*, PFCRT, PFMDR 1and RFLP

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الخلاصة

تشكل الملاريا المقاومة للكلوركوين خطرا متنامياً في الدول المدارية , لذلك فأن استخدام (الموسمات الجزيئية) فى استقصاء مقاومة الطفيل لعقار الكلوركوين قد تشكل أهمية في التغلب على هذه المشكلة .هدفت هذه الدراسة لاستقصاء مقاومة الطفيل(بلازموديوم فلسبارم) لعقار الكلوركوين فى وسط السودان باستخدام الموسمات الجزيئية . شملت هذه الدراسة مائة ستة وسبعون تأكد أنهم موجبون للفحص، تم اختيار أربعة وستون منهم لهذه الدراسة، فقط أربعون تمت متابعتهم كاملا. استخدمت طريقة متابعة حساسية الطفيل في داخل المريض طبقا للجرعة القياسية لفوسفات الكلوركوين وتم استخلاص الحمض النووي الرايبوزي منقوص الأكسجين **(DNA)**من ورقة ترشيح بها دم أخذ قبل بدء العلاج لكل مريض. واستخدمت لتقييم وتحديد مدي انتشار الطفرات المقاومة للكلوركوين في اثنين من مورثات طفيل البلازموديوم فالسبارم **PFCRT K76T** و **PFMDR.1 N86Y** باستخدام جهاز التفاعل السلسلي والإنزيمات القاطعة للحمض النووي . شملت هذه الدراسة أربعون مريضا، استجاب 45% منهم للعلاج بينما لم يستجب 46 % منهم للعلاج وتم علاج كل المرضى الذين لم يستجيبوا للعلاج بعقار الارتميثر أو الكينين .أجري المسح الوراثي للطفرات باستخدام تفاعل السلسلي البلمر(**PCR**)والإنزيمات القاطعة للحمض النووي **(RFLP)** للمورثين **(Pfcrt)** عددها (18) و **(Pfmdr-1)** عددها (29) ووجد أن 72% من جين **(Pfcrt)** تحمل طفرات **(T76)**للجين **(Pfcrt)** وأن 22% بالمورث **(K76 Pfcrt)** بينما وجد أن 6% تحمل طفرات خليط من الاليل الطافر وغير الطافر **(K/T76)**أوضحت نتيجة المسح الوراثي للجين **(Pfmdr-1 Y86N)** بأن 38% تحمل طفرات وأن 55% من الطفيليات لا تحمل طفرة له وأن 7% من الطفيليات تحمل خليط من ألجين الاليل الطافر وغير الطافر. خلصت الدراسة أن زيادة تكرار الجين الطافر **(Pfcrt 76T)**في طفيليات البلازموديوم فالسبارم تعزز افتراضية إمكانية استخدام جين **(Pfcrt 76T)** موسم أساسي لمراقبة ومعرفة حساسية مقاومة طفيل البلازموديوم فالسبارم لعقار للكلوركوين في المسوحات الوبائية .

INTRODUCTION

Resistance to Chloroquine was noted in Thai-Cambodian border and in Colombia since the late 1950s (1), whereas in Africa, the chloroquine resistance was first reported in east of Africa in 1978 and the resistance spread to centre and south of the continent and at last to the west of Africa 1983, and by 1989 chloroquine resistance was wide spread in Sub-Saharan Africa (2).

Polymorphism in two genes of Plasmodium falciparum (P.falciparium) genome had contributed in chloroquine resistance. The P. facibarum chloroquine resistance transporters (Pfcrt) gene located in chromosome 7 and this segment harbors a 13-exon, Pfcrt, having point mutations that associate completely with chloroquine resistant (CQR) in parasite lines from Asia, Africa, and South America (3),

Laboratory studies in Cameroon and Mali have strongly suggested that P. falciparum chloroquine resistance transporter (Pfcrt Lys76Thr) gene can be used as a marker in surveillance for chloroquine-resistant falciparum malaria as a molecular marker (4-6). In diverse geographical areas several studies support the association between the Pfcrt K76T and chloroquine resistance in Africa (Mali) (5), Sudan (7-9), Nigeria (10), Gabon (11), Gambian (12), Malawi (13), Liberia (14), Tanzania (15), Mozambique (16), Ghana (17), Camroon (18) and Senegal (19-20). Asia Thailand (21) and India (22), and Amazon region Brazil (23, 24), however studies from Uganda at the south gate of Sudan reported no association (25).

Initial studies of the gene P. falciparum Multidrug resistance gene (Pfmdr-1) located on chromosome 5, illustrate that an Asn→Tyr mutation at amino acid 86 (N86Y) and other mutations in this gene correlated with chloroquine resistance in {Sudan (7), Mali (5), Gambia (12), Uganda (27), Brazil and Thailand (21)}, however, some authors from {Uganda (28), Brazil (29), and Thailand (30)}, could not find this correlations in their studies.

MATERIAL AND METHODS

Patients: This study was conducted in Marengan Health Center and 48 patients with positive P. Falciparum bloods were enrolled in the study.

The In vivo study:

Chloroquine phosphate (AMIPHARMA Laboratories Ltd. Sudan B N/T0013) was administrated orally at a dose of 10 mg per kilogram (kg) of body weight in day (0) and 5 mg per kg body weight after 6 hours, and in day 1 and day 2, the patients were observed after each dose, and they were given another full dose or a half dose if vomiting occured within 30 min or within 31 min to 1 hour respectively. Clinical and

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parasitological follow-up was done on day 1, 3, 7 and 14. Whenever symptoms were reported or parasiteima increased, the case is considered as in vivo Chloroquine resistance state (31) and alternative treatment was scheduled for that.

Assessment of in vivo response and implementing molecular techniques:

This was done according to WHO assessment (31). Blood samples were obtained on white man filter paper 0.3 immediately before treatment and stored in 4Cº, DNA was extracted from filter paper blood samples by cutting an approximately 4 mm2 piece of paper was placing in 100 ìl methanol, air dried, and incubated at 95 to100 ºC in 50 µl of water for 15 minutes. During incubation, the tube was subjected to high-speed vortex three times. 5 µl of resulting solution used as template for PCR (5)

The PCR reaction was performed in 30 µl volumes which contains 1X PCR Buffer II (Gene Amp® 10X Buffer II [100 mM Tris-HCl pH 8.3, 500 mM KCl], Applied Bio system), 1.5 mM MgCL2 (MgCl2, Applied Bio system), 0.2 mM each of the dNTP (Gene Amp® dNTPs, Applied Bio system), 1.0 µ mol of each sense and antisense primers 1 U of AmpliTag Gold (Applied Bio system), 5 µl of the Template DNA; and the reaction volume was completed to 30 µl by ddH2O.

Nested PCR for the detection of tyr-86 allele of pfmdr-1 in field isolates for all reactions was amplified using Gene Amp® PCR system 9700 (Applied Bio system) in the following conditions: one cycle at 94 ºC for 3 minutes; 40 cycles at 94 ºC for 1 minute, 49 ºC for 1 minute, and 72º C for 1 minute; and a final extension at 72º C for10 minutes was carried out using outer primers MdrA1 and. MdrA3 (32). 3 µl were used for the second round as a template of nested PCR, with anew set of inner primers Mdr A2 and Mdr A4 without change in the PCR condations as described by (32). A nested PCR protocols were used to identify the K76T in the Pfcrt gene (3) with a slight modification on primers used to amplify in Gene Amp® PCR system 9700 (Applied Bio system). The outer primers tcrp- 1 and Pfcrt-13 in the following conditions: one cycle at 94º C for 3 minutes; 40 cycles at 94 ºC for 30 seconds, 55 ºC for 30 seconds, and 60º C for 30 seconds; and a final extension at 60 ºC for 3 minutes. 2 µl of product was diluted 1/20 and 3 µl were used as a template for the nested reaction using Pfcrt-14 and Pfcrt-15 primers in the following conditions: one cycle at 94 ºC for 3 minutes; 40 cycles at 92 C for 30 seconds, 49 ºC for 30 seconds, and 65 ºC for 30 seconds; and a final extension at 65 ºC for 3 minutes. For each series of samples, water was used as a negative control, HB3-strain DNA was used as the wild-type control, and Dd2 DNA was used as the mutant control. All the amplifications were checked using agarose gel electrophoresis, in which PCR products were run in 1.5 %(Pfmdr-1 gene) and 2 %(Pfcrt gene).

Comparing gene sequence in group of individuals; change in one or more base pairs occur causing variation in the sequence, these variations are called polymorphisms, furthermore, restriction site may be created or abolished at that point, this is known as Restriction Fragment Length Polymorphism (RFLP) (33) . The mutations in the tow genes Pfcrt and Pf mdr-1 have been abolished by the restriction site for Apo 1 enzyme (Fig 2.4). Digestion with this enzyme was used for typing these polymorphisms. In a total volume of 15 µl, 2 µl PCR product were digested overnight at 50°C with 1 U Apo I, 1.5 µl 10x NE Buffer 3 (100 mM Nacl, 50 mM Tris-Hcl,10 mM magnesium chloride and 1mM dithiothreitol (pH 7.9 @ 25 °C), 0.15 µl of 100X BSA (200µg/ml) and deionized water. Using the Genomic DNA from strain 3D7 and Dd2 were Amplified and digested in the same way serving as control for complete digestion, and undigested fragment respectively (Fig. 2.1).

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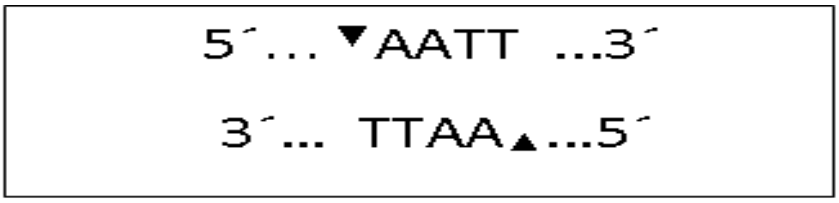

Figure 1 Recognition site for ***Apo 1*** enzyme

Statistical analysis: SPSS (Statistical Package for Social Science) software was used for statistical analysis.

RESULTS

Study area: The study was conducted at Marengan Health Center Medani, Sudan, from September to December 2002, where the malaria incidence is high.

Patients: Patients with symptoms or signs suggesting malaria were screened for the presence of malaria parasites in their peripheral blood. One hundred and seventy six (176) patients were found to be smear positive for malaria; 94% of them were infected with P. falciparum; 6 % with mixed infection P. falciparum and P. vivax or P. vivax only.

Sixty four (64) patients fulfilled the criteria for selection and enrolled in the study; and only 40 patients were completed the period of follow up.

Response of P. falciparum isolates to chloroquine according to in vivo test:

Fig (2) shows the adjustment of isolates to chloroquine according to WHO standard regimen dose and selection criteria. From forty (40) patients treated with chloroquine, only 22 (54%) responded fully with adequate clinical and parasitological response (ACR), in 18 (46%) patients drug resistance was found distributed to 9 (23%) with early treatment failure (ETF) and 9 (23%) with late treatment clinical and parasitological failure (LTF). All the treatment failures were treated with artemether injection 1.6 mg/ Kg body weight or quinine and followed as required, there was no recrudescence reported.

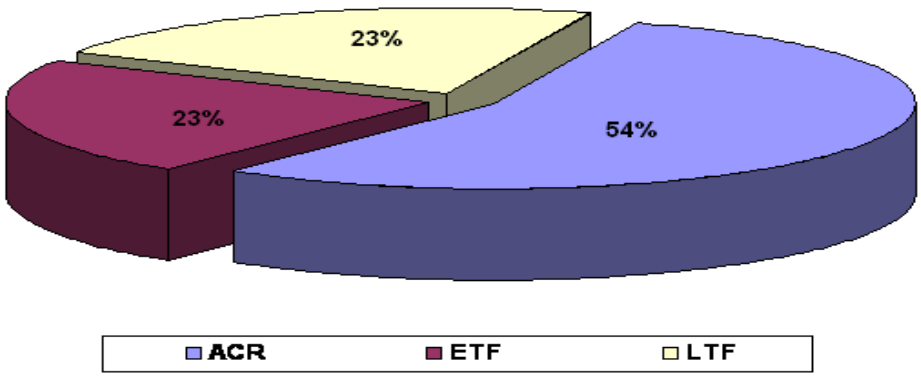

Figure 2: The distribution *P. falciparum* isolates according to *in vivo* sensitivity to chloroquine.

Table 1 shows the distribution of the mean parasitemia and the standard error of in vivo test. According to sensitivity all the 22 (54%), the group of ACR, with mean initial parasitemia, 20089 ± 6520, 5723 ± 2713, and 00 ± 00 in D0, D1, and D3 respectively. Finally, only one patient was found to have severe malaria

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symptoms before day 3, and treated with quinine as alternative drug and followed as required.

Table 1: The parasitaemia mean ± standar error of the mean (S. E. M) according to in vivo sensitivity response to chloroquine

In vivo Sensitivity		Number	Mean ± (S. E. M)
D0	ACR	22	20089 ± 6520
	ETF	9	22550 ± 6532
	LTF	9	16230 ± 7083
D1	ACR	22	5723 ± 2713
	ETF	9	3530± 1180
	LTF	9	5388 ± 3934
D3	ACR	22	00 ± 00
	ETF	8	1869 ± 1093
	LTF	9	00 ± 00
D7	ACR	22	00 ± 00
	ETF	-	-
	LTF	8	1015 ± 541
D14	ACR	22	00 ± 00
	ETF	-	-
	LTF	1	400 ± 00

The prevalence of Pfcrt T76 and Pfmdr 1 mutations:

Fig. (3) Presented the pattern of detection of P. falciparum chloroquine resistance transporter gene (Pfcrt) using nested PCR method where as, (Fig. 4) shows the detection of P. falciparum multi-drug resistance 1 gene using nested PCR method. (Fig. 5), represented the pattern of restriction fragment length polymorphism (RFLP) of Pfmdr 1 gene. The prevalence of Pfcrt T76 and Pfmdr 1 Y86 was screened in 18 and 29 parasite isolates respectively. Restriction fragment length polymorphism (RFLP) technique using Apo 1 enzyme was used for screening the isolates. Table 2 shows that the Pfcrt T76 mutation was present in 13 (72%) samples, 4 (23%) had the wild-type of Pfcrt K76 and only one (5%) sample with mixed alleles of wild and mutant (T76K). and the prevalence of Pfmdr-1 Y86 was present in 11 (38%) mutant type and 16 (55%) were found to be Pfmdr 1 N86 and 2 (7%) carried the mixed alleles of wild and mutant type (Y86N) in (Table 3.).

Figure.3.: Detection of of P. falciparum chloroquine resistance transporter gene (Pfcrt) using nested PCR method.

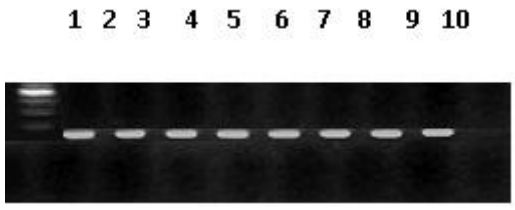

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Fig. 4: Detection of of P. falciparum multi-drug resistance 1 gene using nested PCR method.

Lane 1: M: 100-bp DNA marker. Lane 2: PCR product (positive control). Lanes 3 -11 Positive amplification (502 bp) PCR products and lane 12 negative control.

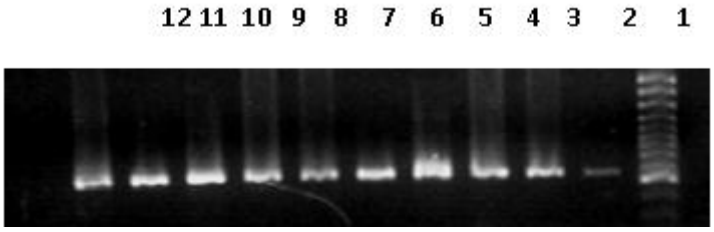


Figure.5: PCR-RFLP screening of P. falciparum isolate for codon 76 polymorphism. Lane 1and 10: DNA markers.

Lane 2: digested PCR product (positive control). Lane 3&5: K76 polymorphism (cutted by Apo1 restriction enzyme to145 and 64 bp). Lane 4, 6, 7, 8&9: T76 polymorphism (uncutted full length 209 Pb).

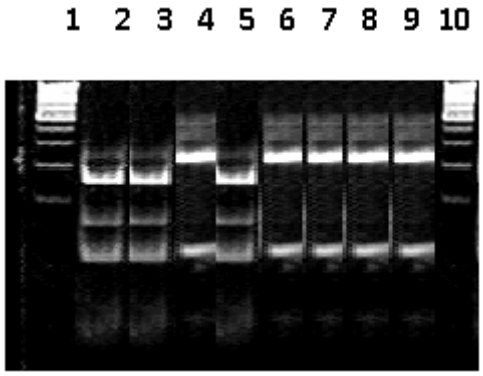
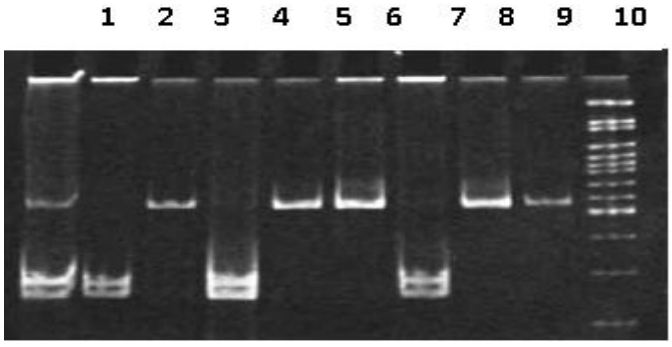


Figure.6: PCR-RFLP screening of P. falciparum isolate for codon 86 polymorphism

Lane 1: Mixed infection by two strains carring both hapoliod alleles N86 and Y86 Lane 2, 4 & 7: N86 allele (cutted by Apo1 restriction enzyme to 254 and 248 bp) Lanes 3, 5, 6 & 8: Y86 allele (uncutted by Apo1 restriction enzyme full length 502 bp). Lane 9: undigested PCR product (positive control). Lane 10: DNA marker.



Association of mutations and treatment outcome:

In (Table 2) six samples were classified as ACR according to in vivo sensitivity test, the molecular technique shows three samples had K76, two of samples had T76 (the mutant type), and the last one with mix alleles T76 and K76. In eight isolates classified as ETF using in vivo test, the molecular techniques showed only one sample (12.5%) which had the wild type K76 and the rest of the isolates (87.5%) carring

the mutant one T 76. In the isolates classified as LTF all isolates (100%) had the mutant type T76. No sample in treatment failure outcome of Pfcrt gene was observed to have mixed alleles.
Pfmdr 1 gene mutation represented in (Table. 3) no sample in the group, which classified as ACR of Pfmdr-1 gene was observed to have mixed alleles and only two isolates with mixed alleles in isolate classified as ETF were found.

Frequency of Pfcrt and Pfmdr 1 genotypes among P. falciparum isolates:

The distribution P. falciparum isolates according to Pfmdr-1 and Pfcrt genotypes were illustrated in (Fig.7)

Table 2: Distribution of Pfcrt genotypes in P.Falciparum isolates according to in vivo sensitivity to chloroquine

Total	Pf Mdr-1 marker			In vivo sensitivity
	N/Y	Y	N	
14	0	4	10	ACR
9	2	3	4	ETF
6	0	4	2	LTF
29	2	11	16	Total

Pfcrt $P = 0.002$ *in vivo* sensitivity $P = 0.000$

Table 3: Distributation of Pfmdr-1 genotypes in P.falciparum isolates according to in vivo sensitivity to chloroquine

Total	**Pfcrt markers**			**In vivo sensitivity**
	K/T	T	K	
6	1	2	3	ACR
8	0	7	1	ETF
4	0	4	0	LTF
18	**1**	**13**	**4**	**Total**

Pf Mdr-1 marker 1 $P = 0.005$

In vivo sensitivity $P = 0.000$

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Table 4: In vivo, and markers marker results for Pfcrt gene and Pfmdr-1 genes in all isolates

Isolate	*In vivo*	Pfmdr 1	Pfcrt
C10	ACR	ND	ND
C11	ACR	ND	ND
C12	ETF	Y	T
C13	ACR	N	K
C15	ACR	N	ND
C16	ETF	N	T
C19	ACR	N	ND
C20	ACR	N	ND
C21	LTF	ND	ND
C22	ETF	ND	ND
C23	ETF	Y	T
C24	ACR	N	K
C25	ACR	Y	ND
C27	ACR	N	ND
C28	ACR	N	ND
C29	LTF	N	T
C30	LTF	Y	T
C31	ACR	ND	ND
C33	ETF	Y	ND
C34	ACR	ND	ND
C35	ACR	ND	ND
C36	ACR	ND	ND
C37	ACR	ND	ND
C38	ETF	N	K
C40	ETF	N/Y	T
C41	ACR	Y	ND
C42	ETF	N	T
C43	ACR	Y	T
C44	ACR	N	ND
C46	LTF	Y	T
C47	LTF	Y	ND
C48	ETF	N/Y	T
C49	LTF	N	T
C50	ACR	N/Y	T/K
C51	LTF	N	ND
C/2R	LTF	ND	ND
C5/R	LTF	Y	T
C52	ACR	N	K
C53	ACR	Y	T
C1/R	ACR	ND	ND
Total	40	29	18

DISCUSSION

In vivo assessment:

In this study, the failure rate for treatment during in vivo assessment of sensitivity to chloroquine was 46%, this is very remarkable because the similar failure rate was reported in Sudan (34), and also similar Frequencies (39-46%) were observed in other African countries (35-38) .

According to the recent classification of WHO criteria for in vivo drug sensitivity into ACR, ETF and LTF (31) , the results distributed as follows; 22 patients (54%) were ACR, 9 patients (23%) ETF and 9 patients were LTF (23%). (Fig. 2).

In (ACR) group, the isolates with low or moderate levels of resistance, the infection could be resolved and cured due to many factors that could be summarized in a proper drug dose, parasites genetic material and partial immunity experiences, this more evident in full immune response patients in endemic areas (39). In case of the (ETF) and (LTF) there are many factors contributing to the treatment outcome; factors that influencing host and parasites; in case of host the main factors are immune status of the patients, for the

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parasites the mutations, which affect drug action, the interaction between sexual stage and the vectors and moreover, the environment may alter in this process (1, 40-42) , all these factors may allow the parasites to survive in the presence of chloroquine.

In general, residents of malaria-endemic areas sometimes spontaneously clear P. falciparum infection without drug treatment, implying an important role for host factors, such as immunity, in this clearance (43).

The effect of the initial parasitemia on the treatment outcome:

Comparison of the effect of the initial parasitemia on the treatment outcome showed no significant difference in chloroquine response at different levels of the initial parasitemia (Table 1), this indicates that; the initial parasitemia has no role in determining the chloroquine in vivo response. This finding was in line with study done in Sennar (44) , however, study done by Wernsdorfer et al, observed higher mean initial parasitemia in the group of resistance parasites, than in sensitive one (45).

Molecular markers:

***P*. falciparum Chloroquine resistance transporter (Pfcrt):**

Characterization of genes controlling drug resistance in parasite, as in dihydrofolates reductease gene lead to the development of molecular markers, this is used for proper diagnosis and early detection of treatment failure (46).

The prevalence of Pfcrt T76 mutations (the mutations which was incriminated in chloroquine resistance) was present in 78% of our isolates, and 22% (4 isolates) carring the wild type of Pfcrt K76 (Fig. 7), most of the isolates with mutation type might have been resistant in vivo, however, 34% and 14% of the mutant alleles T76 and T/K76 were recovered by immune response and classified as ACR (Table 2), this find indicated the important role of the host immunity, this observation was consistent with a number of studies (28, 43, 47).

Most of the isolates classified as in vivo resistance, carrying Pfcrt T76, so based on this above observation; we strongly associated the subsequent resistance to chloroquine in vivo with the presence of Pfcrt T76, at the time of treatment. These data, are in line with genetic evidence of Fidock etal (3), on the other hand, only one isolate was detected carrying the wild type K76, and classified as in vivo resistance (Table 2). The possible explanation is that the patient had mixed infections with low level of resistant parasites carrying the mutant type, under the drug pressure the sensitive parasites were eliminated and the resistant ones expanded resulting in treatment failure. The presence of Pfcrt T76 at the time of treatment was also strongly associated with subsequent resistance to chloroquine in vivo.

***P.* Falciparum multi drug resistance-1 gene (Pfmdr-1):**

The prevalence of the wild type of Pfmdr-1 isolates is more, compared to the mutant type in the screened samples (Fig. 3), indicating the less sensitivity of Pfmdr-1 for detecting the resistance isolates predicted by in vivo test.

Among ACR group, the N86 can predict 71% of the sensitivity in vivo (Table 3), in the resistance group 50% of isolates carrying the mutant type Y86. This observation is consistent with some other studies in which decrease in association between Pfmdr- 1 Y86 and sub-mutations and chloroquine resistance in vivo was reported (48, 49)

Recently, many investigators showed that resistant infections in vivo can be due to parasites with no Pfmdr-1 mutations at position Y86 and gave strong evidence to the link of this resistance to other gene (3, 5, 10).

Chloroquine resistance in Sudan was first reported In the Gezira area (50), and then in Sennar (51) and Gadarif (52), but now resistance to chloroquine was reported in different parts of the country. In the last decade progress has been done in the molecular basis of drug resistance in P. falciparum. As more information on the genetics of anti-malarial resistance becomes available, designing of new molecular-based tools for drug resistance evaluation, and applying them in early detection of drugs resistant will help in proper selection of antimalarial drugs and rapid treatment of patients which is the corner stones in malaria control.

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