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New Haplotypes of the *Plasmodium falciparum* Chloroquine Resistance Transporter (*PFCRT*) Gene Among Chloroquine-Resistant Parasite Isolates

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NEW HAPLOTYPES OF THE *PLASMODIUM FALCIPARUM* CHLOROQUINE RESISTANCE TRANSPORTER (*PFCRT*) GENE AMONG CHLOROQUINE-RESISTANT PARASITE ISOLATES

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Abstract. Mutations in the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene were examined to assess their associations with chloroquine resistance in clinical samples from Armopa (Papua) and Papua New Guinea. In Papua, two of the five *pfcrt* haplotypes found were new: SVIET from Armopa and CVIKT from an isolate in Timika. There was also a strong association ($P < 0.0001$) between the *pfcrt* 76T allele and chloroquine resistance in 50 samples. In Papua New Guinea, mutations in the *pfcrt* gene were observed in 15 isolates with chloroquine minimum inhibitory concentrations (MICs) of 16–64 pmol, while the remaining six isolates, which had a wild-type *pfcrt* gene at codon 76, had MICs of 2–8 pmol. These observations confirm that mutations at codon 76 in the *pfcrt* gene are present in both *in vivo* and *in vitro* cases of chloroquine resistance, and that detection of the *pfcrt* 76T allele could predict potential chloroquine treatment failures.

INTRODUCTION

Chloroquine has been the drug of choice for treating malaria patients for the last 50 years, but the spread of drug-resistant *Plasmodium falciparum* has become a major problem. In Southeast Asia, 21.9 million cases of malaria were reported in 1995 alone.[1,2] Malaria is a serious problem in the eastern islands of Indonesia and in nearby Papua New Guinea. Approximately 20–30% of the population in these regions typically carry malaria parasites at any given time. In addition, 20% of consultations, 16% of hospital admissions, and 14% of hospital deaths are attributable to malaria.[1,2]

Papuan Indonesia (formerly Irian Jaya) and Papua New Guinea have long been plagued by drug-resistant malaria. Resistance to pyrimethamine and chloroquine in the Arso-Waris-Upper Tor River areas of Papuan Indonesia is believed to have arisen in 1959–1961 with the mass distribution of medicated chloroquine and pyrimethamine salts.[3] Resistance of *P. falciparum* to chloroquine was reported from Kalimantan in 1973 and from Papua in 1975.[4,5] An increased risk of chloroquine resistance was reported from the Jayapura region of Papua, Indonesia in the1980s.[6,7] Surveys conducted during the 1990s showed high malaria prevalence rates (60–92%) and levels of chloroquine treatment failure of up to 80% among indigenous and immigrant communities of northern and central Papuan Indonesia.[8–12] However, due to its safety profile, low cost, and relative success in treating mildly symptomatic malaria infections among immune and semi-immune patients, chloroquine remains the treatment of choice for malaria, and no effective alternative strategy has been developed. Molecular markers of drug resistance in *P. falciparum* could prove useful in defining the intensity of resistance in an individual patient and the extent and severity of the problem in communities.

The aim of this study was to examine *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene haplotypes in parasite isolates with known *in vivo* or *in vitro* chloroquine resistance responses.

MATERIALS AND METHODS

The Armopa region is located on the northwestern coast of Indonesian Papua. Prior to Javanese transmigration in 1995, there were small traditional villages and a single health clinic in Armopa. No mass treatment or prophylaxis was practiced before transmigrant arrivals, and the indigenous people of Armopa did not use antimalarial drugs for prophylaxis. However, chloroquine, Fansidar® (pyrimethamine and sulfadoxine) (F. Hoffmann La Roche, Basel, Switzerland), and quinine were presumably available for treatment of clinical malaria. Transmigrants, primarily from malaria-free Java and Bali, had no previous exposure to malaria and no history of antimalarial drug use before settling in Armopa. As per national health standards, they were given chloroquine for self prophylaxis during their first three months after arrival; thereafter, treatment with only chloroquine was provided for uncomplicated cases of clinical malaria. Chloroquine is widely used for treatment of clinical malaria among transmigrants and is dispensed through a health clinic in each settlement.

Blood samples were collected in 1996–1999 from study volunteers who were immigrants to the Armopa SP1 and SP2 sites and had no history of malaria or antimalarial drug use. Patients who were positive for malaria were treated at the health center with chloroquine, Fansidar®, and quinine as the respective first-, second-, and third-line drugs for uncomplicated malaria as per the Indonesian National Health Policy. Chloroquine was given at a dose of 10 mg/kg on the first day, followed by 5 mg/kg 12, 24, and 48 hours later. A single dose of Fansidar® (1 mg/kg of pyrimethamine and 20 mg/kg of sulfadoxine) was given if there was chloroquine treatment failure. This study was carried out after obtaining informed consent from all adult participants and from parents or legal guardians of minors, and was reviewed and approved by the Ethics Committees for Protection of Human Subjects at the Ministry of Health, Republic of Indonesia, the U.S. Navy Medical Research Unit No. 2 (Jakarta, Indonesia), and The

TABLE 1

In vitro chloroquine responses and Plasmodium falciparum chloroquine resistance transporter (pfcrt) gene haplotypes among malaria patients in Armopa, Indonesian Papua*

Patient	Sampling date	In vivo response†	pfcrt codons				
			72	73	74	75	76
CQ004	12/6/96	RI	**S**	V	M	N	**T**
CQ008	12/7/96	RI	**S**	V	M	N	**T**
CQ010	3/10/97	RI	**S**	V	M	N	**T**
CQ010	4/12/97	RIII	**S**	V	M	N	**T**
CQ017	5/2/97	RIII	**S**	V	M	N	**T**
CQ039	1/26/97	RI	**S**	V	M	N	**T**
CQ039	4/24/97	RI	**S**	V	M	N	**T**
CQ041	9/4/97	RIII	**S**	V	M	N	**T**
CQ042	5/8/97	RIII	**S**	V	M	N	**T**
CQ044	4/13/97	RIII	**S**	V	M	N	**T**
CQ044	7/8/97	RIII	**S**	V	M	N	**T**
CQ054	5/3/97	RIII	**S**	V	M	N	**T**
CQ055	5/4/97	RIII	**S**	V	M	N	**T**
CQ060	5/8/97	RIII	**S**	V	M	N	**T**
CQ062	5/14/97	RIII	**S**	V	M	N	**T**
CQ072	12/4/96	RI	**S**	V	M	N	**T**
CQ091	4/16/97	RI	**S**	V	M	N	T
CQ101	1/15/97	RIII	**S**	V	M	N	**T**
CQ118	11/9/97	RII	**S**	V	M	N	**T**
CQ041	8/10/97	RIII	**S**	V	M	N	**T**
CQ041	10/30/97	RIII	**S**	V	M	N	**T**
CQ072	4/21/97	RIII	**S**	V	M	N	**T**
CQ080	5/5/97	RIII	S	V	M	N	**T**
CQ080‡	7/29/97	RI	**S**	V	M	N	**T**
CQ076	10/15/96	RIII	**S**	V	**I**	**E**	**T**
CQ104	5/2/97	RIII	**S**	V	**I**	**E**	**T**
CQ106	11/6/96	RIII	**S**	V	**I**	**E**	**T**
CQ020	3/11/97	RIII	**S**	V	**I**	**E**	**T**
CQ116	5/3/97	RIII	**S**	V	**I**	**E**	**T**
CQ026	3/15/97	RIII	**S**	V	**I**	**E**	**T**
CQ078	8/29/97	RIII	C	V	**I**	**E**	**T**
CQ078	11/26/97	RIII	C	V	**I**	**E**	**T**
CQ077	2/28/97	RIII	C	V	**I**	**E**	**T**
CQ082	11/8/97	RIII	C	V	**I**	**E**	**T**
CQ094	3/23/97	RIII	C	V	**I**	**E**	**T**
CQ113	10/12/97	RII	C	V	**I**	**E**	**T**
CQ118	2/15/97	RIII	C	V	**I**	**E**	**T**
CQ128	3/15/97	RIII	C	V	**I**	**E**	**T**
CQ073	2/3/97	RIII	C	V	**I**	**E**	**T**
CQ024	2/6/97	RIII	C	V	**I**	**E**	**T**
CQ131	2/7/97	RII	C	V	**I**	**E**	**T**
CQ003	1/21/97	RI	C	V	M	N	**T**
CQ059	3/30/97	RI	C	V	M	N	**T**
CQ067	4/30/97	RIII	C	V	M	N	**T**
CQ022	4/4/97	RIII	C	V	M	N	**T**
CQ022	4/23/97	RIII	C	V	M	N	**T**
CQ087	8/1/97	RIII	C	V	M	N	**T**
CQ095	12/13/96	RI	C	V	M	N	**T**
CQ105	4/13/97	RI	C	V	M	N	**T**
CQ036	4/26/97	RII	C	V	M	N	**T**
CQ024	5/6/97	S	C	V	M	N	K
CQ027	2/6/97	S	C	V	M	N	K
CQ036	1/30/97	S	C	V	M	N	K
CQ042	2/4/97	S	C	V	M	N	K
CQ043	9/25/97	S	C	V	M	N	**T**
CQ051	1/24/97	S	C	V	M	N	K
CQ056	4/5/97	S	C	V	M	N	K
CQ091	7/30/98	S	C	V	M	N	K
CQ001	1/26/98	S	C	V	M	N	K
CQ090	1/10/99	S	C	V	M	N	K
CQ062	5/5/98	S	C	V	M	N	K
CQ077	11/28/98	S	C	V	M	N	K
CQ022	2/25/97	S	C	V	M	N	K
CQ095	1/28/98	S	C	V	M	N	K
CQ058	11/13/97	S	C	V	M	N	K
CQ020	11/12/97	S	C	V	M	N	K
CQ060	10/17/98	S	C	V	M	N	K
CQ098	1/28/98	S	C	V	M	N	K
CQ0110	3/15/97	S	C	V	M	N	K
CQ0102	2/3/97	S	C	V	M	N	K
CQ113	7/12/98	S	C	V	M	N	K

TABLE 1 (Continued)

In vitro chloroquine responses and Plasmodium falciparum chloroquine resistance transporter (pfcrt) gene haplotypes among malaria patients in Armopa, Indonesian Papua* (Continued)

* Codon mutations are indicated in bold.
†R = resistant; S = sensitive.
‡ Sample from a patient with a recrudescent parasitemia that was not included in the statistical analysis.

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RESULTS

Of 85 patients, 21 (24.7%) cases cleared their parasitemias within 72 hours, had no recurrence during 28 days of follow-up, and were classified as sensitive to chloroquine. Fifty patients had persistent or recurrent parasitemias and were classified as resistant to chloroquine. Data from 15 patient samples were excluded from analysis due to incomplete clinical histories, intercurrent infections with *P. vivax*, or an inability to amplify gene products. Samples were analyzed for mutations in the *pfcrt* gene after amplification by a polymerase chain reaction (PCR) of DNA extracted from blood samples, followed by restriction fragment length polymorphism (RFLP) analysis and DNA sequencing.[13,14] The DNA from a drug-sensitive strain (D10) was used as a positive control to monitor PCR conditions. As expected, the PCR product was amplified with wild-type alleles of the *pfcrt* gene. No PCR products were amplified in negative controls.

The results of *pfcrt* mutational analysis of samples from 50 cases of chloroquine treatment failure are shown in Table 1. All 50 chloroquine-resistant samples carried the mutant *pfcrt* 76T allele. No mutation was detected at codon 73, but variations were found at codons 72, 74, and 75 in 50 samples: SVMNT (24), CVIET (11), CVMNT (9), and SVIET (6). Statistical analysis (chi-square test with Yates' correction) showed that the *pfcrt* mutation at codon 76 was strongly associated with chloroquine resistance ($P < 0.0001$).[15] Among the 21 chloroquine-sensitive samples, only one carried a mutated *pfcrt* 76 allele.

Analysis of RFLP results from amplification of chloroquine-resistant *P. falciparum* laboratory strains K1, W2mef, VNS, 7G8, a new isolate, 2300, from Timika on the southern coast of Indonesian Papua, and two isolates, F2382 and F1568, from Flores, Indonesia showed mutations in the *pfcrt* gene (Table 2). Seven of these chloroquine-resistant laboratory strains showed three different *pfcrt* haplotypes with mutations at codons 74, 75, and 76: CVMNT (7G8), CVIKT (2300), and CVIET (K1, W2 mef, VNS, F2382, and F1568). The wild-type haplotype CVMNK was found in the chloroquine-sensitive control strain D10.

The Wosera region of East Sepik province in Papua New Guinea is highly endemic for malaria. Mutation analysis of the genes involved in chloroquine resistance from Papua New

TABLE 2

In vitro chloroquine responses and *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene haplotypes among laboratory strains and field samples*

Strains/samples	Origin	MIC_{50}†	*pfcrt* codons				
			72	73	74	75	76
Laboratory strains							
D10	Papua New Guinea	1	C	V	M	N	K
2300	Papua	75	C	V	**I**	**K**	**T**
K1	Thailand	130	C	V	**I**	**E**	**T**
W2 mef	Southeast Asia	100	C	V	**I**	**E**	**T**
VNS	Vietnam	80	C	V	**I**	**E**	**T**
F2382	Flores, Indonesia	30	C	V	**I**	**E**	**T**
F1568	Flores, Indonesia	128	C	V	**I**	**E**	**T**
7G8	South America	300	C	V	M	N	**t**
Field samples (from all Papua New Guinea)							
DR1		64.0	**S**	V	M	N	**T**
DR3		64.0	**S**	V	M	N	**T**
DR9		32.0	**S**	V	M	N	**T**
DR21		32.0	**S**	V	M	N	**T**
DR24		32.0	**S**	V	M	N	**T**
DR5		16.0	**S**	V	M	N	**T**
DR12		16.0	**S**	V	M	N	**T**
DR15		16.0	**S**	V	M	N	**T**
DR22		16.0	**S**	V	M	N	**T**
DR2		32.0	**S**	V	M	N	**T**
DR11		64.0	C	V	M	N	**T**
DR4		32.0	C	V	M	N	**T**
DR18		64.0	C	V	M	N	**T**
DR20		16.0	C	V	M	N	**T**
DR23		16.0	C	V	M	N	**T**
DR14		2.0	C	V	M	N	K
DR17		2.0	C	V	M	N	K
DR19		8.0	C	V	M	N	K
DR10		2.0	C	V	M	N	K
DR13		2.0	C	V	M	N	K
DR16		2.0	C	V	M	N	K

* Codon mutations are indicated in bold.
† For the laboratory strains, 50% mean inhibitory concentration (MIC_{50}) values are in nanomoles and were determined by an *in vitro* microtiter assay.[31] For the field isolates, values are in picomoles and were calculated at the Australian Army Malaria Institute.

Guinea has shown the presence of *P. falciparum* isolates carrying the *pfcrt* SVMNT haplotype, which is usually found in South American parasites, but not the CVIET haplotype of Southeast Asian isolates.[16] The results of mutational analysis of 21 samples of *P. falciparum* obtained from malaria patients in Papua New Guinea are shown in Table 2. Fifteen of these samples with chloroquine minimum inhibitory concentrations (MICs) between 16 and 64 pmol had the 76T allele. However, six isolates with MICs of 2–8 pmol had the wild-type *pfcrt* allele at codons 72, 73, 74, 75, and 76. There were three *pfcrt* haplotypes among the 21 Papua New Guinea samples (Table 2). The wild-type haplotype CVMNK was observed in isolates with MIC values of 2–8 pmol, while the chloroquine-resistant *pfcrt* haplotypes CVMNT and SVMNT were observed in samples with MICs between 16 and 64 pmol.

DISCUSSION

Although chloroquine resistance in *P. falciparum* has been reported in Indonesia and Papua New Guinea since the early 1970s, molecular analysis and *in vitro/in vivo* responses to antimalaria drugs have only recently been determined in this region. This study analyzed the association of mutations in

TABLE 3

Geographic distribution of *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene haplotypes among chloroquine-resistant strains of *P. falciparum**

pfcrt codons					Chloroquine susceptibility	Location
72	73	74	75	76		
C	V	M	N	K	Sensitive	
C	V	M	N	**T**	Resistant	Papua, Papua New Guinea, South America
C	V	**I**	**K**	**T**	Resistant	Papua
C	V	**I**	**E**	**T**	Resistant	Papua, Southeast Asia, Africa
S	V	**I**	**E**	**T**	Resistant	Papua
S	V	M	N	**T**	Resistant	Papua, Papua New Guinea, South America

* Codon mutations are indicated in bold.

the *pfcrt* gene in samples of *P. falciparum* that had been characterized as chloroquine sensitive or resistant by *in vitro* or *in vivo* tests.[14] A strong association between mutations in the *pfcrt* gene and chloroquine resistance ($P < 0.0001$) was observed in those samples from individuals who had chloroquine treatment failure *in vivo* or had displayed chloroquine MICs of 16–64 pmol in the *in vitro* test.

Studies to elucidate the molecular and biochemical mechanism of resistance to chloroquine have been in progress for more than a decade. Chloroquine resistance in *P. falciparum* involves decreased accumulation of the drug. However, the precise mechanism is not known.[17] Mutations in the *P. falciparum* multidrug resistance 1 (*pfmdr1*) gene were implicated and involvement of at least two genes was hypothesized; mutations in both the *pfcrt* and the *pfmdr1* genes appear to be necessary for resistance to chloroquine.[13,18,19] A mutation in the *pfcrt* gene (located on chromosome 7) at codon 76, with a change from lysine to threonine, has been invariably found in chloroquine-resistant strains and also in chloroquine-resistant field samples from Laos, Cameroon, Mozambique, Uganda, and South America.[13,20–27] Transformation of chloroquine-sensitive isolates with the Dd2 *pfcrt* gene sequence containing the 76T mutation consistently produced chloroquine-resistant clones, and insertion of the wild-type *pfcrt* gene caused resistant isolates to exhibit sensitivity to chloroquine.[13] Studies on field isolates have shown the occurrence of three haplotypes of *pfcrt* gene alleles: CVMNK among chloroquine-sensitive isolates, CVIET among chloroquine-resistant isolates from Southeast Asia and Africa, and SVMNT among chloroquine-resistant isolates from South America and Papua New Guinea.[13,16,28] The presence of the 76T *pfcrt* gene mutation has been correlated with risk of therapeutic failure when malaria due to *P. falciparum* is treated with chloroquine.[22,29,30] Recently, an analysis of the genetic mutations associated with chloroquine resistance in an area highly endemic for malaria (the Wosera region of East Sepik province in Papua New Guinea) was also reported.[16] All (100%) samples from treatment failures (Indonesian Papua) and 67% of the isolates (Papua New Guinea) collected prior to treatment in the *in vitro* studies carry the mutated *pfcrt* allele 76 (Tables 1 and 2).

In this study, analyses of known laboratory isolates that are resistant to chloroquine showed the presence of a mutation in the *pfcrt* gene. Samples collected in Papua New Guinea for *in vitro* chloroquine susceptibility testing provide further support for our data from clinical studies in Armopa. Although a mutated *pfcrt* codon 76 is invariably present in chloroquine-resistant isolates, comparison of *pfcrt* haplotypes revealed some interesting features. In both Armopa (Papua) and Papua New Guinea, the CVMNK haplotype was the wild type. In Papua New Guinea, the chloroquine-resistant haplotypes detected were SVMNT and CVMNT, as demonstrated in other studies (Table 3).[16,28] Interestingly, the *pfcrt* haplotypes CVIET (African, Southeast Asian) and SVMNT (South American) were also detected in Papuan samples. In addition, two new *pfcrt* haplotypes were detected in Papua that have not been previously reported: SVIET, which was found in clinical samples isolated from cases of treatment failure in Armopa and CVIKT, a haplotype found in chloroquine-resistant laboratory strain 2300, which was isolated in 1985 in Timika, Papua. The presence of African, South American, Southeast Asian, and two new chloroquine-resistant haplotypes in these regions raises the question of the evolution of these five haplotypes. They may have evolved by sequential mutations of the gene in this region, where the parasite is widely circulated, or the parasites with these haplotypes were transferred into this region. This speculation on the origin of haplotypes awaits detailed studies with data from other loci in the genomes of *P. falciparum* isolates.

In conclusion, our results support the hypothesis that the molecular basis of chloroquine resistance involves mutations in the *pfcrt* gene and that detection of a mutated *pfcrt* allele 76 could predict potential chloroquine treatment failures.

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