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Origin and Dissemination of Chloroquine-Resistant *Plasmodium falciparum* with Mutant *pfcrt* Alleles in the Philippines

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The *pfcrt* allelic type and adjacent microsatellite marker type were determined for 82 *Plasmodium falciparum* isolates from the Philippines. Mutant *pfcrt* allelic types P1a and P2a/P2b were dominant in different locations. Microsatellite analysis revealed that P2a/P2b evolved independently in the Philippines, while P1a shared common ancestry with Papua New Guinea chloroquine-resistant parasites.

Mutations in the *pfcrt* gene have been associated with chloroquine resistance in *Plasmodium falciparum* (4, 6, 8, 12). Fifteen amino acid mutations at positions 72, 74, 75, 76, 97, 144, 148, 160, 194, 220, 271, 326, 333, 356, and 371 have been identified in *pfcrt* of chloroquine-resistant (CQR) parasites from various regions (3, 7–9, 15). In general, the CQR isolates from Southeast Asia and Africa have *pfcrt* alleles with seven to nine mutated codons that are linked to a pattern of C/***I***/***E***/***T***/H(***L***)/A(***F***)/L(***I***)/L/I(***T***)/***S***/***E***/***S***/T(***S***)/***T***/***I*** (boldface italics indicate the mutated codons), from positions 72 to 371, while the CQR parasites from South America and Papua New Guinea (PNG) possess *pfcrt* alleles with four or five mutated codons forming a pattern of ***S***(C)/M/N/***T***/H/A/L/L/I/***S***/Q/***D***/***T***/***L***/R (3, 4, 8, 9, 15). Our previous study identified novel *pfcrt* allelic types in parasites from Morong, the Philippines, with four or five mutated amino acids linked into a pattern of ***S***(C)/M/N/***T***/H/***T***/L/***Y***/I/A/Q/***D***/T/I/R. In vitro chloroquine susceptibility testing indicated that these parasites with the novel *pfcrt* allelic types were CQR (3). Among the mutated codons, A144T and L160Y were exclusively identified in the Philippine isolates, suggesting that CQR *pfcrt* alleles evolved independently in the Philippines (3). The novel Philippine *pfcrt* allelic types were named P2a and P2b according to the nomenclature used by Wellems and Plowe (3, 14).

In this study, we analyzed *pfcrt* allelic types in *P. falciparum* isolates from several locations within the Philippines to ascertain the geographical distribution of these novel and other *pfcrt* allelic types. The ancestral origin of the observed CQR *pfcrt* allelic types was investigated by microsatellite marker analysis (15).

Eighty-two *P. falciparum* isolates were collected from the following locations in the Philippines between 1989 and 2002 (Fig. 1): Morong (n = 20) (3), Bagac (n = 4), and Dinalupiham (n = 12) in Bataan Province, Central Luzon; Puerto Princesa, Palawan (n = 12), and Tayabas (n = 6) in Quezon Province, South Luzon; Conner (n = 10) and Kabugao (n = 5) in Kalinga-Apayao Province, Northern Luzon; and Agusan del Sur, Mindanao (n = 13). Genomic DNA was extracted using a Wizard Plus Minipreps DNA Purification System (Promega),

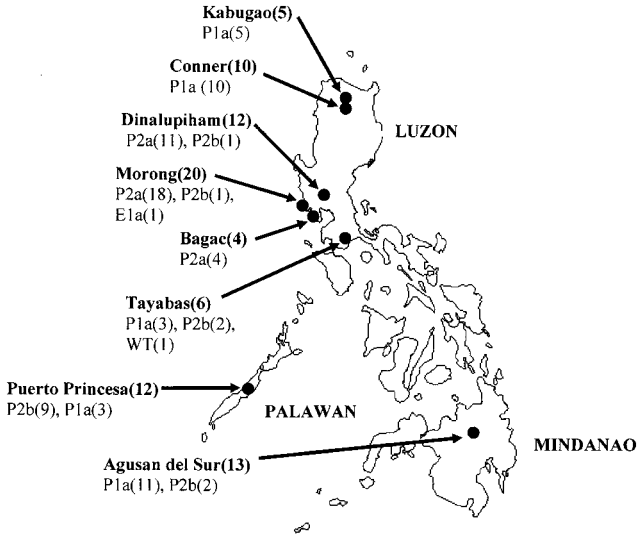

FIG. 1. Locations in the Philippines where parasite isolates were examined for *pfcrt* allelic types and microsatellite marker types. Numbers in parentheses following the name of a district/municipality indicate the total number of parasite samples. *pfcrt* allelic types and numbers detected at each location are indicated below the name of each district/municipality.

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TABLE 1. *pfcrt* allelic types and polymorphisms in microsatellite markers flanking *pfcrt* in *P. falciparum* samples collected from different districts/municipalities in the Philippines compared to those from other areas

Origin (total no. of isolates) and sample	No. of isolates	%	MS[b] marker size (bp)		PFCRT (allelic type)[a]	MS marker size (bp)		
			B5M77 (−20 kb)	2F10 (−5 kb)		PE12A (+6 kb)	2H4 (+22 kb)	PE14F (+106 kb)
Philippines								
Morong (20)								
PH1 group	18	90	149	182	CMNTHTYAQDLR (P2a)	314	228	136
PH2	1	5	149	182	SMNTHTYAQDIR (P2b)	314	228	136
PH4	1	5	149	170	CIETHALSESTI (E1a)	314	204	145
Bagac (4), B1 group	4	100	149	182	CMNTHTYAQDLR (P2a)	314	228	136
Dinalupiham (12)								
D1 group	10	83	149	182	CMNTHTYAQDLR (P2a)	314	228	136
D16	1	8	149	182	CMNTHTYAQDLR (P2a)	314	220	142
D3	1	8	149	182	SMNTHTYAQDIR (P2b)	314	228	136
Palawan (12)								
PAL2 group	5	42	149	182	SMNTHTYAQDIR (P2b)	314	228	136
PAL12 group	2	17	149	182	SMNTHTYAQDIR (P2b)	314	228	151
PAL7	1	8	149	182	SMNTHTYAQDIR (P2b)	314	228	148
PAL6	1	8	149	182	SMNTHTYAQDIR (P2b)	314	220	136
PAL5 group	2	17	147	194	SMNTHALSQDLR (P1a)	328	228	136
PAL1	1	8	147	194	SMNTHALSQDLR (P1a)	328	228	148
Tayabas (6)								
T1 group	2	33	149	174	SMNTHALSQDLR (P1a)	328	218	148
T4	1	17	149	174	SMNTHALSQDLR (P1a)	328	184	148
T2 group	2	33	149	182	SMNTHTYAQDIR (P2b)	314	228	136
T3	1	17	149	176	CMNKHALAQNIR (CQS)	328	182	142
Mindanao (13)								
L104 group	4	31	149	174	SMNTHALSQDLR (P1a)	328	184	148
L088 group	2	15	149	174	SMNTHALSQDLR (P1a)	328	184	151
L016	1	8	149	174	SMNTHALSQDLR (P1a)	328	184	142
M013 group	2	15	149	174	SMNTHALSQDLR (P1a)	328	206	148
M007	1	8	149	174	SMNTHALSQDLR (P1a)	328	186	148
X039	1	8	149	174	SMNTHALSQDLR (P1a)	328	204	148
X098	1	8	149	182	SMNTHTYAQDIR (P2b)	314	202	148
X097	1	8	149	182	SMNTHTYAQDIR (P2b)	314	202	151
Connor (10)								
C3 group	7	70	149	174	SMNTHALSQDLR (P1a)	328	184	148
C1 group	3	30	149	174	SMNTHALSQDLR (P1a)	328	228	136
Kabugao (5), Kab1 group	5	100	149	174	SMNTHALSQDLR (P1a)	328	184	148
Other areas								
Brazil (1), 7G8	1		151	190	SMNTHALSQDLR (W1a)	314	194	142
Thailand (5)								
Dd2 group	2		149	170	CIETHALSESTI (E1a)	314	204	145
K1	1		149	170	CIETHALSESII (E1b)	314	204	145
TM93-C1088	1		149	170	CIETLALSESTI (E1c)	314	204	145
C2B	1		149	170	CIETHALSESTI (E1a)	314	184	148
Solomon (4)								
N18 group	2		149	174	SMNTHALSQDLR (P1a)	328	184	142
N70 group	2		147	174	SMNTHALSQDLR (P1a)	328	184	142
PNG (6)								
AN001 group	4		149	174	SMNTHALSQDLR (P1a)	328	184	142
AN018 group	2		149	174	SMNTHALSQDLR (P1a)	328	192	139

[a] Amino acid positions in PFCRT shown are 72, 74, 75, 76, 97, 144, 160, 220, 271, 326, 356, and 371. *pfcrt* allelic types E, W, and P refer to resistant loci originating in the eastern and western hemispheres and in the Pacific, respectively (3, 14).

[b] MS, microsatellite.

and DNA fragments covering the 12 known mutations in *pfcrt* were amplified by PCR (3). PCR products were purified with a NucleoSpin extraction kit (Macherey-Nagel) and sequenced. Microsatellite markers flanking the *pfcrt* gene, B5M77(−20 kb), 2E10 (−5 kb), PE12A (+6 kb), 2H4 (+22 kb), and PE14F (+106 kb), were amplified and analyzed as previously described (15).

Polymorphisms in *pfcrt* in *P. falciparum* collected from the Philippines are shown in Table 1. Novel *pfcrt* allelic types P2a and P2b with mutations at A144T and L160Y were identified in 18/20 and 1/20 Morong isolates (Central Luzon) (3), respectively. P2a was also the major *pfcrt* allelic type in Bagac (4/4) and Dinalupiham (11/12) in Central Luzon, while P2b was the major type in Puerto Princesa, Palawan (9/12). In comparison, P2b was only identified in a small proportion of the isolates from Tayabas (2/6) in Southern Tagalog and from Agusan del Sur, Mindanao (2/13). Neither P2a nor P2b was detected in isolates from Conner and Kabugao, Northern Luzon, where P1a, the PNG allelic type, dominated. The chloroquine-sensitive wild-type *pfcrt* was detected in one isolate from Tayabas. Overall, among the 82 Philippine isolates analyzed, 48 (58.5%) possessed the P2a/P2b alleles, 32 (39%) had the P1a allele, and 1 (1.2%) each had the E1a allele and the wild type.

Genotyping using *msp1*, *msp2*, and *glurp* (10) was performed to examine the heterogeneity of the isolates on loci not linked with *pfcrt*. Various banding patterns were observed among isolates with identical novel P2a and P2b *pfcrt* allelic types, indicating the isolates were diverse rather than clonal (data not shown). This is highlighted in 18 Morong isolates, where nine distinct banding patterns identified for *msp1*, *msp2*, and *glurp* corresponded to the P2a *pfcrt* allele.

The dissemination of mutant *pfcrt* allelic types in the Philippines is shown in Fig. 1. P2a was the major type in isolates from Central Luzon, while P2b predominated in those from Palawan. P1a was found to be dominant in isolates from Northern Luzon and Mindanao. Although geographically the provinces of Bataan and Quezon are situated in Luzon, they are well separated by mountain ranges. This may provide an explanation for the dominance of P2a and P1a, respectively, in these provinces. Palawan, though separated by sea, shares a closely related dominant *pfcrt* allelic type with Central Luzon. The novel mutations in P2a and P2b alleles (A144T and L160Y) were not found in a wild-type *pfcrt* gene, indicating that these mutations in the CQR isolates were not inherited from chloroquine-susceptible parasites in the Philippines. In addition, none of the Philippine isolates with A144T and L160Y mutations ($n = 48$) carried the A220S mutation very commonly seen in CQR parasites elsewhere. The mutually exclusive presence of A144T/L160Y with A220S suggests that A144T and L160Y may play a similar role as A220S in CQR.

Five microsatellite markers flanking the *pfcrt* gene, B5M77, 2E10, PE12A, 2H4, and PE14F (15), were analyzed for all the Philippine isolates included in this study, as well as several standard laboratory lines and isolates from Southeast Asia, Oceania, and South America. As shown in Table 1, all isolates with the P2a *pfcrt* allele, except for one from Dinalupiham (D16), gave the identical size for all five microsatellite markers, and the banding pattern was unique compared to that in parasites from other regions. While some isolates with the P2b *pfcrt* allele had microsatellite markers identical to those in P2a, others showed variations in some microsatellite markers. The unique microsatellite marker patterns of P2a/P2b *pfcrt* allelic types demonstrate that P2a/P2b alleles evolved independently in the Philippines. The similarity between the microsatellite patterns of P2a and P2b and fact that P2b carried an extra mutation (C72S) suggest that the P2b allelic type was derived from P2a. We hypothesize that CQR parasites (P2a) originated in Central Luzon and spread south to Palawan and Mindanao with the addition of an extra mutation in *pfcrt* (P2b). This is consistent with field observation of chloroquine resistance in Philippines: first documented in Luzon from the early 1970s to the 1980s (2, 5, 11, 13) and subsequently in Palawan (1).

In contrast, the microsatellite marker patterns of the Philippine isolates with P1a *pfcrt* type closely resembled that of the PNG and the Solomon Islands isolates, suggesting that P1a *pfcrt* alleles in parasites from the Philippines and Oceania evolved from a common origin and may have spread from neighboring countries. The only Philippine isolate with the E1a *pfcrt* type (PH4) was identical to Dd2 (Thailand) in microsatellite marker pattern (Table 1). Significantly, this was obtained in close proximity to the site of the main transit camp for Indochinese refugees in the Philippines.

This study confirms that at least two founder events of chloroquine resistance have occurred in the Pacific region, one in PNG and one in the Philippines, and demonstrates that under chloroquine selection pressure, *P. falciparum* parasites with various genetic backgrounds have developed chloroquine resistance independently by mutating different positions in the *pfcrt* gene.

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