Genetic Evidence for *Plasmodium falciparum* Resistance to Chloroquine and Pyrimethamine in Indochina and the Western Pacific between 1984 and 1998


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Abstract. *Plasmodium falciparum* resistance to chloroquine and pyrimethamine is widely distributed in malaria-endemic areas. The origin and geographic spread of this drug resistance have been inferred mainly from records of clinical resistance (treatment failure). Identification of the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene and the dihydrofolate reductase (*dhfr*) gene as target genes of chloroquine and pyrimethamine, respectively, has made it possible to trace the history of genetic resistance to these two drugs. However, evidence for genetic resistance has been limited because of scarcity of archival specimens. We examined genotypes of *pfcrt* and *dhfr* in Indochina (Thailand, Myanmar, and Laos) and the Western Pacific (the Philippines, Indonesia, and Papua New Guinea) between 1984 and 1998 by testing samples obtained from malaria cases imported to Japan. Results show that 96% (28 of 29) and 77% (20 of 26) of samples had resistant genotypes of *pfcrt* and *dhfr*, respectively, substantiating the inferred history of clinical resistance in these geographic areas during this period.

INTRODUCTION

Drug resistance imposes a serious burden in the treatment of *Plasmodium falciparum* malaria in tropical regions. Chloroquine (CQ) resistance in *P. falciparum* first appeared almost simultaneously in Southeast Asia and South America in the late 1950s.1 In Southeast Asia, failure of CQ treatment was reported in all countries in Indochina during the 1960s, with CQ resistance rapidly reaching the Western Pacific and Africa by the early 1970s. CQ resistance is currently prevalent in all malaria-endemic areas, except the Caribbean, China, and the Middle East.

Sulfadoxine-pyrimethamine (SP) was used as an alternative drug for treatment of CQ-resistant malaria in Indochina in the late 1960s.2 However, resistance to SP rapidly emerged in malaria-endemic areas where CQ was replaced with SP.4 In Thailand, SP was used as a first-line treatment in the early 1970s,4,6 but it was abandoned in the early 1980s because of increased treatment failures.6 Laos and Myanmar also introduced SP in the early 1970s, but stopped its usage recently.6 SP was rarely used in Cambodia, Vietnam, and Malaysia for treatment of CQ-resistant malaria, but resistance to SP has also been prevalent in these countries.4,6 Resistance to SP was also observed in the Western Pacific countries of Indonesia, Papua New Guinea, and the Philippines in the 1980s.4 Thus, SP resistance is now highly prevalent in Indochina and the Western Pacific.2

The molecular basis of CQ and pyrimethamine (Pyr) resistance has been well characterized in *P. falciparum*. The target gene of CQ resistance is the *P. falciparum* CQ resistance transporter (*pfcrt*) gene, which encodes a putative transporter localized in the digestive vacuole membrane of the parasite. Among 10 amino acid substitutions within *pfcrt* identified among drug-resistant parasites, replacement of lysine with threonine at amino acid 76 is essential for conferring resistance to CQ.5 The target gene of Pyr is the dihydrofolate reductase (*dhfr*) gene, which encodes dihydrofolate reductase, a key enzyme in the folate biosynthetic pathway. Six amino acid substitutions within *dhfr* have been reported among Pyr-resistant parasites.9,10 An essential mutation for resistance is a substitution of serine with asparagine at amino acid 108.10,11 Additional point mutations (at 16, 51, 59, and 164) are associated with increased levels of Pyr resistance *in vitro*.10,11 Sequence polymorphisms occur in the *pfcrt* and *dhfr* loci in field isolates of *P. falciparum*, and distributions of polymorphisms differ among geographic areas.12–15 Confining the polymorphisms to Indochina and the Western Pacific, a *pfcrt* genotype of CVIET at positions 74, 75, and 76 (mutated residues are underlined) represents the most common CQ-resistant type in Indochina, and a *pfcrt* genotype carrying SVMNT at positions 72 and 76 is observed predominantly in the Western Pacific.12,13 In the Pyr-resistant *dhfr* polymorphism, triple (CIRNI at 51, 59, and 108) and quadruple (CIRNL at positions 51, 59, 108, and 164) mutants are prevalent in Indochina, and the double mutant (CNRNI) is the major *dhfr* genotype in the Western Pacific.14,15 Migration of genotypes conferring resistance to CQ and Pyr from Indochina to Africa have also been demonstrated.16,17

Previous evidence for drug resistance of *P. falciparum* has been derived from records of clinical resistance (treatment failure) and/or *in vitro* drug sensitivity tests. Obtaining genetic evidence for resistance to CQ and Pyr was not feasible until after the identification of target genes and mutation(s) involved in the drug resistance.2 It should be emphasized that genetic resistance and clinical resistance are not always consistent because selection of drug-resistant parasites results from the interplay of the parasite, drug, and human host, and is largely influenced by immune factors and the pharmacokinetics of the drugs.18,19

In areas highly endemic for malaria, such as tropical Africa, CQ and Pyr are still effective, although only partially, in persons infected with drug-resistant genotypes largely because of their immunity, which is acquired after repeated infec-

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of imported malaria in Japan were approximately 100 during the period, extending back before genetic resistance was first reported in these areas, and substantiating the inferred history of clinical resistance in these geographic areas during this period.

In this study, we investigate polymorphisms in the \textit{pfcrt} and \textit{dhfr} loci in Indochina and the Western Pacific between 1984 and 1998 using archival samples. Results obtained show a high prevalence of resistance to CQ and Pyr during this period, extending back before genetic resistance was first reported in these areas, and substantiating the inferred history of clinical resistance in these geographic areas during this period.

\textbf{MATERIALS AND METHODS}

\textbf{Parasite samples.} Blood samples used in this study were collected as part of a national surveillance system of imported malaria cases in 50 hospitals in Japan between 1984 and 1998, and stored as blood smears. All cases were diagnosed by examination of Giemsa-stained blood smears by two experienced microscopists. The total number of samples examined was 588, with 30–60 samples per year. Annual reported cases of imported malaria in Japan were approximately 100 during this period.23 Among the 588 cases, 229 samples were positive for \textit{P. falciparum}, 347 for \textit{P. vivax}, 7 for \textit{P. ovale}, and 5 for \textit{P. malariae}. Of the 229 \textit{P. falciparum} samples, we examined 55 single \textit{P. falciparum} infections that originated in Southeast Asia and the Western Pacific in this study. The remaining 174 cases from Africa, South Asia, and South America will be analyzed elsewhere.

\textbf{Extraction of DNA.} Parasite DNA was extracted according to the method of Kimura and others.24 Briefly, Giemsa-stained slides were dipped in xylene and then in methanol to remove the immersion oil and dye. Each blood smear was scraped off a destained slide with an edge of a clean glass slide, and subjected to DNA purification using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). To avoid cross-contamination of parasite DNA sample by sample, each scraping off was done using a fresh glass slide on a plastic dish, which were disposed immediately after scraping off. A non-infected blood smear was also used as a negative control in the DNA extraction and polymerase chain reaction (PCR) amplification procedures. The purified DNA, eluted in 100 µL of elution buffer provided with the kit, was stored at 4°C.

\textbf{Polymerase chain reaction.} We amplified a 190-basepair region in the second exon of \textit{pfcrt}, which contained the polymorphic regions of amino acid residues 72–76, by using the nested PCR method described by Djimde and others.25 Primers were the same as those previously described.25 For amplification of \textit{dhfr}, we targeted three regions of approximately 190 basepairs that covered four polymorphic residues involved in Pyr resistance. The PCR amplification was performed using Phusion™ high-fidelity DNA polymerase (New England Biolabs, Beverly, MA) in a 50-µL reaction mixture containing 1 µL of extracted DNA, 1 µL (10 mM) of each dNTP mixture (0.2 mM each), 10 µL of 5x Phusion HF buffer, 0.5 µL of DNA polymerase, and 0.5 µM of primers described below.

\textbf{RESULTS}

\textbf{Polymorphism in pfcrt.} Of the 55 samples subjected to PCR, amplification of the \textit{pfcrt} fragment was successful for 29 samples. The low rate of successful amplification was probably caused by low parasite numbers in the blood smears, many of which had parasitemias < 0.03%. Thus, the success rate was 64% for samples with parasitemias > 0.03%, and 33% for those samples with parasitemias < 0.03%, which is consistent with a low rate of successful amplification using blood smear samples with low parasitemias.24 The polymorphisms observed are shown in Table 1. Records of previously reported \textit{pfcrt} genotypes are combined with the present results and shown in a time-line scheme (Figure 1A). All of our samples showed a CQ-resistant \textit{pfcrt} genotype, with the exception of a sample isolated from the Philippines in 1985. The CVIET CQ-resistant \textit{pfcrt} genotype was present in samples from Myanmar and Laos collected in 1994. This date is five years earlier than the first reported record of this CQ-resistant \textit{pfcrt} polymorphism in those two countries.6

In samples from Thailand, the CVIET genotype was detected in 1984, 1991, and 1992. An earlier presence of this resistant genotype has been noted in some culture-adapted parasite strains isolated from Thailand: the K1 strain isolated in 1979,26 the Indochina III strain in 1984,27 and the TM284 strain in 1990.28 The prevalence of this CQ-resistant genotype was 100% in 1995 in Thailand.6,28 Thus, our results suggest...
the persistence of this resistant genotype in Thailand from at least 1979 to the present time. The CVIDT polymorphism, a variant of CVIET, was reported in Cambodia in 2001 and 2004, and was also detected in two of our 1998 samples from Laos and/or Thailand. These findings suggest an earlier presence of this resistant genotype in central Indochina (Laos/Thailand/Cambodia) than previously reported. The Papua New Guinea form of CQ-resistant \( \text{pfcrt} \) genotype (CVMNK), was not detected in our limited samples from Indochina.

In the Western Pacific countries (Indonesia, Papua New Guinea, and the Philippines), most samples (n = 16) showed the CQ-resistant \( \text{pfcrt} \) polymorphism SVMNT. This genotype was detected in a sample from Indonesia from 1991. This date is much earlier than previous records from Indonesia collected in 1999 and 2002. We identified another \( \text{pfcrt} \) genotype (CVMNN) from Indonesia in 1986 and it is reported that this CVMNN mutant exhibits resistance to CQ \textit{in vitro}. In Papua New Guinea, all samples (n = 8) isolated between 1986 and 1998 showed the SVMNT \( \text{pfcrt} \) genotype, which is consistent with the report by Mehlotra and others. In contrast, in samples collected from Papua New Guinea between 1956 and 1965, all carried the wild-type \( \text{pfcrt} \) genotype.

### Table 1

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* Values in parentheses indicate number of samples. Mutated residues are in \textbf{bold} and \textit{underlined}. \( \text{pfcrt} \) = \textit{P. falciparum} chloroquine resistance transporter; \( \text{dhfr} \) = dihydrofolate reductase; ND = not done.

† Persons visited both countries.
‡ Mixed infection of two distinct \( \text{pfcrt} \) genotypes.

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Papua New Guinea form of CQ-resistant \( \text{pfcrt} \) genotype (CVMNK), was not detected in our limited samples from Indochina.

In the Western Pacific countries (Indonesia, Papua New Guinea, and the Philippines), most samples (n = 16) showed the CQ-resistant \( \text{pfcrt} \) polymorphism SVMNT. This genotype was detected in a sample from Indonesia from 1991. This date is much earlier than previous records from Indonesia collected in 1999 and 2002. We identified another \( \text{pfcrt} \) genotype (CVMNN) from Indonesia in 1986 and it is reported that this CVMNN mutant exhibits resistance to CQ \textit{in vitro}. In Papua New Guinea, all samples (n = 8) isolated between 1986 and 1998 showed the SVMNT \( \text{pfcrt} \) genotype, which is consistent with the report by Mehlotra and others. In contrast, in samples collected from Papua New Guinea between 1956 and 1965, all carried the wild-type \( \text{pfcrt} \) genotype. Our results, together with these records, suggest that the SVMNT genotype, which appeared before 1982, persisted until 1998 (and probably until now). In samples from the Philippines, the SVMNT genotype was detected in samples from 1985, six years earlier than the first record of this type in 1991. We also detected the wild-type (CVMNK) \( \text{pfcrt} \) genotype in samples from the Philippines in 1985 and 1998. A substantial parasite population showed the wild-type \( \text{pfcrt} \) genotype present in 1997, which suggested the persistence and co-
prevalence of both CQ-resistant and CQ-sensitive parasite populations in the Philippines.

**Polymorphism in dhfr.** Of the 29 samples sequenced for \( pfcrt \), 26 samples were successfully sequenced for three \( dhfr \) fragments, encompassing four polymorphic sites. Results are shown in Table 1 and Figure 1B. The triple mutant Pyr-resistant \( dhfr \) genotype CIRNI was present in samples from Thailand collected in 1984 (n = 2). This triple mutant \( dhfr \) is identical to the genotype of the Indochina III strain in 1984 (Figure 1).27 Thus, the CIRNI genotype was already prevalent as early as 1984 in Thailand. In Laos, the double mutant Pyr-resistant \( dhfr \) genotype CNRNI was first reported in 1999.14 In our study, this double mutant was detected in isolates from Laos from 1994 and 1998 (n = 4), which suggested the presence of this double mutant in Laos at least five years earlier than previously recorded. In Myanmar, one sample isolated in 1997 was wild-type (CNCSI). However in 1999, \( dhfr \) polymorphism reportedly consisted of 90% Pyr-resistant genotypes (CNRNI, CNRNL, and CIRNL).14 It is not known whether a Pyr-resistant \( dhfr \) genotype was present in Myanmar before 1999.

In the Western Pacific, the triple mutant \( dhfr \) genotype was not found, but the wild-type, single, and double mutant \( dhfr \) genotypes were detected. Wild-type \( dhfr \) was present between 1985 and 1990 in Papua New Guinea (n = 3) and the Philippines (n = 2). The Pyr-resistant \( dhfr \) (single mutant) genotype CNCNI was detected in samples from Indonesia, Papua New Guinea, and the Philippines between 1985 and 1986, and also in one isolate from Papua New Guinea in 1998. The double mutant \( dhfr \) (CNRNI) was obtained from samples from Indonesia in 1991, Papua New Guinea in 1987, and the Philippines in 1985. These dates are much earlier than the first record of this double mutant type in 1996 from Indonesia and Papua New Guinea.39,40 In addition, the presence of these Pyr-resistant \( dhfr \) mutant genotypes have not been previously reported in the Philippines.

**DISCUSSION**

The aim of this study was to obtain genetic evidence of \( P. falciparum \) drug resistance to CQ and Pyr in Indochina and the Western Pacific between 1984 and 1998, during which time reports of \( pfcrt \) and \( dhfr \) genotypes have been limited.35,36 Our results obtained with archival samples present genetic evidence of resistance of this parasite to CQ and Pyr during this period. Most of our samples (96%, 28 of 29) had a CQ-resistant \( pfcrt \) genotype, and there was a clear geographic separation of two resistant genotypes: CVIET in Indochina and SVMNT in the Western Pacific (Figure 1). Ge-
nomic resistance to Pyr was somewhat lower in frequency (77%, 20 of 26) than CQ resistance. The fact that Pyr was introduced in Indochina approximately 20 years later than CQ for treatment failure of *P. falciparum* malaria is consistent with the late spread of Pyr resistance in these areas.\(^{1,4}\) Also, single, double, and triple mutants of *dhfr* were detected. This situation reflects the present distribution of *dhfr* polymorphism in these areas (Figure 1).

These results of genetic evidence for drug resistance are generally consistent with the history of clinical resistance in Indochina and the Western Pacific.\(^{32,40}\) Thus, the present study has substantiated a widely distributed idea that treatment failures were ascribed to genetic resistance to these drugs in Indochina and the Western Pacific. We were unable to find an association of genetic resistance with clinical resistance in our samples because records of drug treatment were accessible to only two malaria patients: one who traveled to Thailand in 1992, and the other who traveled to Papua New Guinea in 1998 (Table 1). The first patient cured after receiving quinine, and the second patient, who had parasites of the *CVIET* type CQ-resistant *pfcrt* genotype and the *CIRN* type Pyr-resistant *dhfr* genotype, died after being treated with quinine and SP.

Additionally, when combined with results of previous reports, our study has three interesting findings. First, a CQ-resistant *pfcrt* genotype (SVMNT) was co-prevalent with a CQ-sensitive genotype in the Philippines in 1998, which is consistent with a relatively high prevalence (30%) of the CQ-sensitive *pfcrt* genotype in 1997.\(^{29}\) Treatment with CQ is still effective in more than half of *P. falciparum*-infected patients in this country.\(^{34}\) Notably, the persistence of this CQ-sensitive *pfcrt* genotype is in sharp contrast to countries, such as Thailand, the Solomon Islands and Vanuatu,\(^ {6,44,45}\) where there is 100% prevalence of the CQ-resistant *pfcrt* genotype. Second, the *CVIET pfcrt* genotype was present in 1998 in the Philippines. Together with reports showing the same type in 1991 and 2002,\(^ {57,38}\) this finding suggests the persistence of this resistant type throughout the 1990s in the Philippines. This CQ-resistant form of *pfcrt* was also reported in Indonesia in 1999 and 2002.\(^ {32,33}\) It remains to be clarified whether the *CVIET* genotype originated independently in the Western Pacific or was imported from Indochina.\(^ {28}\) Third, the *CVMNN pfcrt* genotype was present in Indonesia. One field isolate of this type was reported from Indonesia in 2002.\(^ {33}\) Importantly, the *CVMNN* mutant, which was obtained from *in vitro* culture under CQ pressure, showed resistance to CQ.\(^ {34}\) The *pfcrt* mutant carrying N at residue 76, an amino acid change other than T that results in CQ resistance, can occur in field isolates.

The efficacy of Pyr in treatment of persons with *P. falciparum* infections and the prevalence of both wild-type and Pyr-resistant genotypes of *dhfr* currently vary in the countries of Indochina and the Western Pacific.\(^ {14,15,40,42}\) Together with these reports, our present finding of the three resistant *dhfr* genotypes (single, double, and triple mutants), as well as the wild-type genotype, between 1984 and 1998 may reflect different histories of the use of Pyr in these areas. Thailand is the only country that introduced SP as a first-line treatment in the mid 1970s in Asia.\(^ {5,6}\) However, *in vivo/in vitro* resistance to SP reached 100% in the 1980s.\(^ {7}\) As a result, the drug policy of Thailand was then switched to mefloquine as a first-line treatment in the mid 1980s.\(^ {6}\) We identified *dhfr* triple mutants in two Thai samples collected in 1984, which is consistent with the change of drug policy in the mid 1980s. We did not detect the *dhfr* quadruple mutant, which currently accounts for the highest population of *dhfr* mutants in Thailand,\(^ {15}\) in our archival samples. The quadruple mutant was first identified in samples collected from Thailand in 1995.\(^ {14}\) We report of *dhfr* polymorphism in the Philippines, which shows the wild-type and resistant-type *dhfr* genotypes (single and double mutants) in the 1980s and 1990s.

In conclusion, our analysis of archival samples shows genetic evidence for a wide distribution of *P. falciparum* resistance to CQ and Pyr in Indochina and the Western Pacific during the 1980s and 1990s. It also sheds light on the history of drug resistance in these areas, supporting previous records of clinical resistance during this period.

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