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Is the resistance transporter gene *pfcrt* linked with susceptibility of *Plasmodium falciparum* to chloroquine?

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Abstract

Recent studies have definitively demonstrated a link between mutations in the gene *pfcrt* and resistance to the antimalarial chloroquine (CQ) in *Plasmodium falciparum*. Although these mutations are predictive of CQ resistance they are not quantitatively predictive of the degree of resistance. We had undertaken analysis of a total of 95 recently adapted *P. falciparum* isolates from Thailand. Parasites had been characterized for their drug susceptibility phenotypes and genotypes with respect to *pfcrt.* From the original 95 isolates, 20 were selected for complete *pfcrt* sequence analysis. Almost all of the parasites characterized carried the previously reported mutations K76T, A220S, Q271E, N326S, I356T and R371I. On complete sequencing, isolates were identified with novel mutations at K76A and E198K. The isolates carrying E198K were less resistant to CQ than those that were not. Data from the present study suggested that other genes may also contribute to the degree of resistance once the resistance phenotype is established through mutations in *pfcrt* gene.

Keywords*: Plasmodium falciparum; pfcrt, pfmdr1,* quinoline antimalarials, drug resistance

Introduction

Multidrug resistant *Plasmodium falciparum* including resistance to structurally related antimalarial aminoquinolines such as chloroquine (CQ), quinine (QN) and mefloquine (MQ), is still problematic along the border areas of Thailand, especially Thai-Myanmar border. Reduced dug accumulation is a phenotypic feature of CQ resistance (CQR) which can be partially reversed by the calcium channel blocker verapamil, an agent that also reverses CQ resistance. Two genes have been linked with this phenotype namely *pfmdr1* and *pfcrt*. The *pfmdr1* gene is located on chromosome 5 and *pfcrt* gene is located on chromosome 7. The weight of molecular evidence suggests that while *pfmdr1* may exert a modulatory effect in parasite susceptibility to CQ (1), mutation in *pfcrt* is the principal determinant of CQR (2). Although it is generally accepted that PfCRT is the principal determinant of CQR, it is not possible to predict the degree of CQR based on the *pfcrt* genotype alone or even in combination with *pfmdr1* genotype. It is clear form our own surveillance studies and those of others that parasites considered to be CQR in actual fact, display a broad range of sensitivity to the drug. In this study we have characterized the phenotype-genotype relationship between a total of 95 recently adapted isolates of *P. falciparum* from Thailand. Furthermore, 20 selected isolates were fully sequenced for *pfcrt* gene, in order to look for novel mutations that might be implicated in the degree of CQR.

Methods

In vitro **drug susceptibility testing:** A total of 95 *P. falciparum* field isolates were collected from malaria endemic area of Thailand. All were adapted to continuous culture

according to the methods of Trager and Jensen (3) with modifications. Laboratory strains of G112 (CQ-sensitive) and K1 (CQ-resistant) clones were used as positive control. Susceptibilities of *P. falciparum* isolates to CQ, QN, and MQ were assessed using the radio-isotopic technique based on the uptake of [^{3}H]Hypoxanthine (4). IC_{50} value (drug concentration that inhibits the parasite growth by 50%) was used as an indicator for antimalarial susceptibilities of the tested drugs, and was determined from a log-dose response curve plotted using the Grafit™ computer program (Erithacus Software Ltd., U.K). Sensitivity of the isolates to CQ, QN and MQ was categorized based on IC_{50} values described by Cerutti *et al*, (5).

Detection of *pfcrt* and *pfmdr1* polymorphisms: Genomic DNA was extracted using Chelex-resin (Biorad Co. Ltd., USA). Previously published nested and PCR/RFLP methods were employed to detect *pfmdr1* at the codons 86 (6), and *pfcrt* mutations at the codons 76, 220, 271, 326, 356 and 371 (2). The primers and reaction conditions used were according to the previously described methods (2).

***Pfcrt* DNA sequencing:** The *pfcrt* gene was amplified from genomic DNA using a nested PCR strategy. Exons 1-2, 3-8 and 9-13 were sequenced independently using the primers designed by our group. Each amplicon was then cloned and the positive clones were picked from white or blue colonies following overnight incubation in selection media, S-Gal (Sigma, USA). The plasmid DNA was purified and digested with the restriction enzyme *EcoRI* to confirm the correct insertion. Finally, sequencing was carried out using the M13 forward and reverse primers and DNA*star* (Lasergene) was utilized for sequence analysis.

Statistical analysis: All data are expressed as mean ± standard deviation. Data were assessed for normality using a Shapiro-Wilk statistical test. A Kruskal-Wallace statistical test was utilized to assess the relationship between mutations in *pfcrt* and *pfmdr1* and drug IC_{50}. Correlations were assessed by Spearman Rank test.

Results

***In vitro* drug susceptibility testing:** A total of 30 (32%), 61 (64%), and 4 (4%) isolates were categorized as highly CQ-resistance, moderately CQ-resistance, and CQ-sensitive, respectively [geometric mean (95% CI) IC_{50}: 130.9 (122.1-140.2), 60.8 (56.7-65.3), and 23.0 (20.5-25.5) nM, respectively] (Figure 1A). For QN, 1 (1%), and 92 (99%) isolates were categorized as QN-resistant and QN-sensitive, respectively [geometric mean (95% CI) IC_{50}: 654.4 and 144.4 (129.4-161) nM, respectively]. For MQ, 44 (32%), and 47 (68%) isolates were categorized as MQ-resistant and MQ-sensitive, respectively [geometric mean (95% CI) IC_{50}: 38.2 (34.4-42.4), and 13.3 (11-16) nM, respectively] (Figure 1B).

Genetic polymorphisms: Genotyping for *pfcrt* revealed a 100% prevalence of the 76T, 220S, 271E, 326S and 371I mutations (95 of 95 samples) with a 94% prevalence of the 356T Mutation (89 of 95 samples). The K1-type mutation of the *pfmdr1* was seen in only 5% (5 of 95) of the samples with almost all isolates containing the wild type Asn at position 86.

Novel sequence polymorphisms in *pfcrt*: The sequencing results, one isolate carried the novel mutation at codon 76 with a different amino acid substitution K76A. This is a unique position 76 mutation, which has never been reported before in field isolates. Four of the field isolates categorized as displaying moderate CQ resistance carried a E198K mutation which may be functionally relevant.

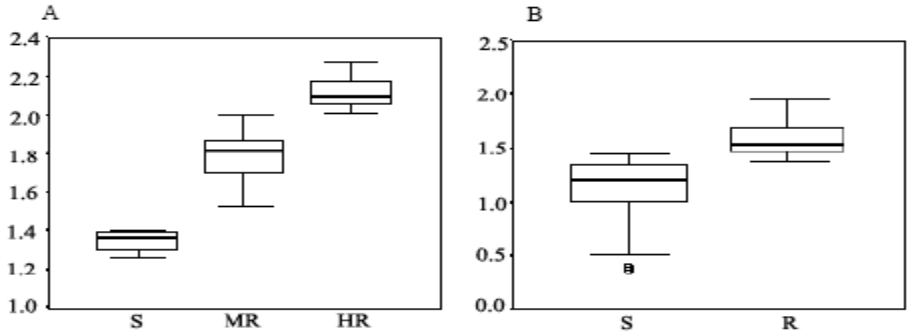

Figure 1 Box plot between logIC_{50} and the order of drug susceptibility. (A) CQ was categorized into highly resistant (HR: IC_{50} > 101 nM), moderately resistant (MR: 30.9 < IC_{50} ≤ 100.9 nM) and sensitive (S: IC_{50} ≤ 30.9 nM). (B) MQ was categorized into 2 groups, resistant (R; IC_{50} >24 nM and sensitive (S; IC_{50} ≤ 24 nM).

Discussion

The K76T mutation of *pfcrt* is reported to be highly associated with CQ-resistance. The frequency of this mutation is almost 100% in Thailand. Consequently there seems to be no link between the frequency of this gene mutation and CQ sensitivity. Parasites carrying the K76T mutation with comparable patterns of other *pfcrt* gene mutations still showed a variable degree of CQ-resistance. Data reported from other South East Asian countries, *i.e*., the Philippines, showed different mutations carrying K76T and N326D without 220S residue, together with the novel mutations A144T and L160Y (7). Furthermore, four novel mutations A144F, L148I, I194T and T333S were observed from Cambodian isolates (8). These mutation patterns were not observed in Thailand. From our results, revealed the novel 76 haplotype K76A in one isolate. Previously, the substitution of other amino acids at this codon, *i.e*., K76I and/or K76N has been reported only in laboratory strains. This allelic type replaces lysine (positively charged amino acid) with alanine (non-charged amino acid), similar to the charge loss found with K76T, K76I and K76N mutations. In addition, the new mutation at position E198K was detected in 4 out of 20 isolates with high CQ sensitivity (low IC_{50}). These findings may support the proposed hypothesis that CQR results from a "charged drug leak", in which the loss of positive charge in the channel of PfCRT might allow the protonated species of CQ to leak out of the digestive vacuole, thus reducing vacuolar CQ concentration and ultimately conferring resistance (9).

Conclusion

Data from the present study suggest that, apart from the reported mutations of *pfcrt* gene--K76T, A220S, Q271E, N326S, I356T and R371I, other genes may also contribute to the degree of resistance once the resistance phenotype is established through mutations in *pfcrt* gene.

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