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SHORT REPORT: POLYMORPHISMS IN THE CHLOROQUINE RESISTANCE TRANSPORTER GENE IN PLASMODIUM FALCIPARUM ISOLATES FROM LOMBOK, INDONESIA

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Abstract. The polymorphisms in the Plasmodium falciparum multidrug resistance 1 (pfmdr1) and P. falciparum chloroquine resistance transporter (pfcrt) genes, which are associated with chloroquine resistance, were examined in 48 P. falciparum isolates from uncomplicated malaria patients from the West Lombok District in Indonesia. The point mutation N86Y in pfmdr1 was present in 35.4% of the isolates and mutation K76T in pfcrt was found in all but one of the samples studied. Identified pfcrt haplotypes were mainly identical to the Papua New Guinea type S_agtVMNT (42 of 48, 87.5%), and a few isolates had the Southeast Asia type CVIET (5 of 48, 10.4%). Moreover, one P. falciparum isolate harbored the K76N mutation, giving rise to the haplotype CVCMNN, which was not previously reported in field isolates. Our findings suggest that chloroquine resistance in this area might have the same origin as in Papua New Guinea.

The mechanism of chloroquine (CQ) resistance in Plasmodium falciparum has been investigated and mutations in the P. falciparum CQ resistance transporter gene (pfcrt) located on chromosome 7, and the P. falciparum multidrug resistant gene 1 (pfmdr1), located on chromosome 5, have been implicated. The substitution of threonine for lysine in codon 76, K76T in the pfcrt gene, was shown in vitro to be associated with CQ resistance in isolates from Asia, Africa, South America, and Papua New Guinea. Sequence polymorphisms at position 72-76 of this gene have been associated with the geographic origin of parasite samples, with the CVIET pattern in resistant isolates from Asia and Africa, and with SVMNT in resistant isolates from Papua New Guinea and South America. The multidrug resistant gene pfmdr1 with a mutation of asparagine to tyrosine at position 86 (N86Y) has been associated with in vitro resistant strains. Although its participation is not clear, it has been suggested that the pfmdr1 mutation may confer some advantage to the parasite in the presence of CQ, thus increasing the level of CQ resistance. Furthermore, a recent study that included samples from four countries of Southeast Asia described the mutations N86Y in pfmdr1 and K76T in pfcrt genes as molecular markers for predicting clinical outcome of CQ treatment.

In Indonesia, the first cases of resistance were reported in the early 1970s from Kalimantan and Irian Jaya. Although resistance has been reported on several islands in Indonesia, with resistance as high as 95% for P. falciparum and 84% for P. vivax, CQ continues to be the first-line treatment of P. falciparum and P. vivax malaria because of its safety and availability at very low cost. Here we examined the prevalence of polymorphisms in the pfmdr1 and pfcrt genes in 48 P. falciparum isolates from the West Lombok District of Indonesia. In addition, the possible origin of CQ resistance in Indonesia is discussed.

Blood samples were collected from 48 patients with uncomplicated P. falciparum malaria in sub-district Batulayar in West Lombok in the West Nusa Tenggara Province of Indonesia (Figure 1) from June to September 2002. Sub-district Batulayar has a population of approximately 35,658. The climate is tropical and malaria transmission occurs more frequently during dry season between April and October, although low-level transmission occurs throughout the year, especially in the hilly-forested ranges of Sidemen to Pusuk. Cases of P. falciparum and P. vivax malaria and a few cases of P. malariae malaria have been reported in the area. The recommended first-line treatment is CQ, 25 mg/kg given over a three-day period. When a treatment failure occurs, the combination of sulfadoxine/pyrimethamine is prescribed. Inclusion criteria were a fever ≥37.5°C during the last 48 hours and a positive result in the NOW ICT® (Binax, Portland, ME) rapid malaria test. Blood samples were collected on filter paper and transported to the Meninting district health center laboratory for malaria testing. Samples positive for P. falciparum malaria were processed thereafter in Japan at the Department of Protozoology of Nagasaki University. Informed consent was obtained from each individual. The study was reviewed and approved by the ethical committee of the Institute of Tropical Medicine of Nagasaki University and the executive committee of the Malaria Control Project in Lombok and Sumbawa under the Japanese International Cooperation Agency partnership program.

The DNA was extracted from filter paper by cutting the blood spot into pieces and soaking them in 0.5% saponin in HBS buffer (140 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.2). Thereafter, the QIAamp DNA Kit (Qiagen, Valencia, CA) was used according to the manufacturer’s instructions. The parasite lines FCR3 and K1 were used as controls for the detection of polymorphism at position 86 in pfmdr1 and direct sequencing analysis of pfcrt gene. For genotyping of the glutamate-rich protein (glurp) gene, strains K1 and 3D7 were used as controls in the amplification.

To determine the presence of tyrosine at position 86 in pfmdr1, a nested polymerase chain reaction (PCR)–restriction fragment length polymorphism protocol was used as previously described. Digestion with the restriction endonuclease Apo I (New England Biolabs, Inc., Beverly, MA) detects tyrosine at position 86 in pfmdr1 and direct sequencing analysis of pfcrt gene. For genotyping of the pfmdr1 and pfcrt genes, strains K1 and 3D7 were used as controls in the amplification.
PCR purification kit (Qiagen) and directly sequenced on an ABI310 automated sequencer using ABI PRISM Big Dye Terminator Cycle kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions.

The **glurp** gene, located on chromosome 11, which has a high degree of polymorphism, was assessed for evaluation of diversity of the *P. falciparum* isolates population in the region. The amplification products were resolved by electrophoresis on a 1% agarose gel and stained with ethidium bromide. The **glurp** amplification product sizes were estimated using DNAnug software (John Nash, Institute for Biologic Sciences, National Research Council of Canada, Ottawa, Ontario, Canada). For comparisons, Fisher’s exact test was used.

Seventeen isolates (35.4%) had the 86Y mutation in the *pfmdr1* gene. 26 had wild type N86, and 5 carried both alleles (Table 1). In previous studies in Irian Jaya and West Papua, N86Y was found to show a correlation with clinical resistance to CQ and as a molecular marker had a sensitivity of 93% and a specificity of 82%. Both point mutations in the *pfmdr1* and *pfcrt* genes have been proposed to indicate a tendency toward reduced susceptibility to CQ. Thus, our results suggest potential CQ resistance in the region, although other factors may influence the final treatment outcome. The combination of geographic remoteness to health facilities and lack of interest in seeking medical attention driven by both financial reasons and lack of knowledge resulted in an overall follow-up rate of 23% (11 of 48). After 14 days of CQ treatment, 5 of 11 patients were not able to clear the parasites, and had a tendency to harbor 86Y in the *pfmdr1* gene (Table 1). However, a larger sample size is required to obtain conclusive results.

A new mutation, K76N, which substitutes asparagine for lysine, was found in one isolate from the sub-village Pusuk, generating the haplotype CVMNN. For confirmation of this finding, independent PCR amplifications and at least three repetitions of sequencing were carried out. In all cases, unambiguous electropherograms were obtained, showing AAT that codes for asparagine at position 76. The K76N mutation could be misidentified as a K76T substitution by a PCR-restriction enzyme protocol. To our knowledge, this is the first time that K76N has been reported in a field study. However, it has been reported in laboratory experiments after exposure of parasites to lethal concentrations of CQ. In those experiments, Cooper and others demonstrated that the K76N mutation confers the verapamil-reversible CQ-resistance phenotype associated with greatly reduced accumulation of the drug. Contrary to those in vitro experiments, the patient possessing this rare *pfcrt* haplotype cleared parasites after treatment with CQ. Since other factors participate in the clinical outcome, it would be interesting to look for more isolates with CVMNN and carry out the in vitro susceptibility test.

The sequence analysis of codons 72-76 in the *pfcrt* gene (Table 1) allowed identification of previously reported haplotypes *SVMNT* (42 of 48, 87.5%) and *CVIET* (5 of 48, 10.4%). The *pfcrt* SVMNT haplotype with serine coded by AGT has been found in Bougainville, Papua New Guinea. The sequence analysis of codons 72-76 in the *pfcrt* gene (Table 1) allowed identification of previously reported haplotypes *SVMNT* (42 of 48, 87.5%) and *CVIET* (5 of 48, 10.4%). The *pfcrt* SVMNT haplotype with serine coded by AGT has been found in Bougainville, Papua New Guinea. The sequence analysis of codons 72-76 in the *pfcrt* gene (Table 1) allowed identification of previously reported haplotypes *SVMNT* (42 of 48, 87.5%) and *CVIET* (5 of 48, 10.4%). The *pfcrt* SVMNT haplotype with serine coded by AGT has been found in Bougainville, Papua New Guinea.
the main island of Papua New Guinea, and East Timor. The haplotype CVIET has been reported in countries of Southeast Asia. Since Lombok, Indonesia is located near Papua New Guinea (Figure 1), it is not unexpected that both the SVMT and CVIET haplotypes were detected.

Furthermore, in our attempt to evaluate the diversity among the isolates studied, we assessed the glurp gene and 11 glurp genotypes were found in West Lombok, ranging from 450 to 1,100 basepairs. The West Lombok District, despite its small area, shows a high degree of diversity in the P. falciparum population that might be a product of high rate of transmission of malaria or human transmigration.

Upon examination for any linkage among the alleles studied in the pfmdr1, pfcrt, and glurp genes, significant associations were found between the pfcrt CVIET haplotype and pfmdr1 86Y (P = 0.0193), the CVIET haplotype and glurp 450 (P < 0.001), and pfmdr1 86Y and glurp 450 (P = 0.0057). Our findings showed that the majority of isolates have pfcrt haplotype SVMT, pfmdr1 86N, and glurp with molecular masses greater than 450 homogeneously distributed in all the villages from West Lombok, indicating that these might be indigenous in the area. A few isolates harboring pfcrt haplotype CVIET, pfmdr1 86Y, and glurp 450, found mainly in Kedondong Atas village, were most likely introduced recently. Therefore, CQ resistance in Lombok might have the same origin as the Papua New Guinea strains, and the Southeast Asian pfcrt haplotype CVIET might have been introduced only recently in a particular region. Further studies are being carried out in isolates from Lombok and other Indonesian islands to determine the prevalence of the novel K76N mutation and its association with clinical outcome in vivo susceptibility to CQ.

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