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Journal of Clinical Microbiology, 48(1), pp.70-77; 2010

Longitudinal survey of *Plasmodium falciparum* infection in Vietnam: Characteristics of antimalarial resistance and their associated factors.

Running title: Molecular epidemiology of malaria in Vietnam

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ABSTRACT

Plasmodium falciparum (*P. falciparum*) is responsible for the main cause of human malaria and one of the important pathogens related to high morbidity and mortality. The total number of malaria patients in Vietnam has gradually decreased over the last decade. However, the spread of pathogens with drug resistance remains a significant problem. Defining the trend in genotypes related to drug resistance is essential for malaria control in Vietnam.

We undertook a longitudinal survey of *Plasmodium falciparum* malaria during 2001-2002 and 2005-2007. The *pfcrt*, *pfmdr1*, *pfdhfr* and *pfdhps* genes were analyzed by sequencing, and correlations among study year, age, gender and genotype were identified statistically. The ratio of chloroquine resistant genotype *pfcrt*76T was found to have decreased rapidly subsequent to 2002. A high number of mutations in the *pfdhfr* and *pfdhps* genes were only observed in 2001-2002, while the emergence of the parasites with a new K540Y mutation in *P. falciparum* dihydropteroate synthetase (PfDHPS) was observed in 2002. Males and those in the younger age brackets demonstrated a correlation with vulnerability of malaria infection and the strain with *pfcrt*76K or that with decreased number of mutations in *pfdhfr* and *pfdhps*. The parasites with *pfcrt*76T exhibited a greater

number of mutations in *pfdhfr* and *pfdhps*.

INTRODUCTION

Plasmodium falciparum has long been one of the most important pathogens, causing severe illness and death worldwide. In the 1990s, more than one million people living in Vietnam suffered from malarial infections, resulting in thousands of deaths per year. Since this time, the National Institute of Malariology, Parasitology and Entomology (NIMPE), and the government of Vietnam have focused a great deal of time and effort into a malaria control program. As a result, the incidence of malaria in 2003 was only 12% of that reported in 1992 (1). However, the spread of drug resistant isolates, including multidrug resistant strains, has become a critical problem in Vietnam and has led to significant failure in treatment. Thus a further understanding of the incidence of malaria cases with detailed parasite genotype information and the identification of factors relating to the acquisition of drug resistant isolates may prove important for the determination of effective and economical treatment choices in clinical settings.

The *Pfcrt* gene is located on chromosome 7 and encodes the vacuolar membrane transporter protein *P. falciparum* chloroquine resistant transport (PfCRT) (21). While several point mutations associated with chloroquine resistance have been determined previously, substitution of K for T in codon 76 has been shown to be specifically related to

resistance *in vitro* (21, 23). The allelic variation of *pfcrt*-related drug resistance differs among various geographical areas. The *pfcrt*72-76, CVIET, CVIDT and SVMNT variants are prevalent in the Indochinese Peninsula (13, 21, 24). *Pfmdr1* is a gene located on chromosome 5, which encodes P-glycoprotein homologue 1 (Pgh1). This protein is localized to the digestive vacuole membrane where it is thought to function in the import of solutes, including some antimalarial drugs, into the digestive vacuole (21). *Pfmdr1* mutations in codons 86, 184, 1034, 1042 and 1246 have been reported previously, and have been shown to correlate with susceptibility to chroloquine, quinine or mefloquine (23). Sulfadoxine-pyrimethamine (SP) resistance is thought to be due to specific point mutations in the *pfdhfr* and the *pfdhps* gene. The *pfdhfr* gene encodes dihydrofolate reductase (DHFR), the target enzyme of pyrimethamine or trimethoprim. Conformational changes in this enzyme due to point mutations result in the prevention of adequate drug access. The codon positions in the *pfdhfr* gene that are related to resistance include 16, 50, 51, 59, 108, 140 and 164 (23). The deduced pathway for the resistant mutants suggested that all multiple mutants emerged through stepwise selection from the single mutant S108N (18). The *pfdhps* gene encodes the enzyme dihydropteroate synthetase (DHPS). Point mutations in this gene also lead to conformational changes in DHPS, and result in resistance to

sulfadoxine and sulfamethoxazole. The loci responsible for resistance have been identified at positions 436, 437, 540, 581 and 613 (23).

In Vietnam, chloroquine resistant *P. falciparum* was reported in the 1960s for the first time (12, 14). Ngo et al. reported in 2003 that all of the isolates acquired from 18 adult rubber plantation workers residing in southern Vietnam demonstrated the *pfcrt* 76T mutation (14). In contrast, Phuc et al. reported that the mutant prevalence was only 38.5% when investigating 39 malaria patients in the Quang Tri Province of central Vietnam (15). While malaria strains resistant to antifolates have continued to increase in prevalence since the 1980s. Masimirembwa et al. analyzed 40 *P. falciparum* isolates obtained from malaria patients, and reported that 97.5% demonstrated a *pfdhfr* mutation that was related to pyrimethamine resistance, while 95.0% demonstrated a *pfdhps* mutation that was associated with sulphadoxine resistance (12).

In the 1990s, treatment for malaria mainly involved mono-therapy with artemisinin or single dose combinations of mefloquine with artemisinin or artesunate in Vietnam. However, the recrudescence rates of these treatment regimens were as high as 25%. As a result, the Vietnamese Ministry of Health introduced CV8 treatment containing dihydroartemisinin, piperaquine, trimethoprim and primaquine as part of the National

Malaria Control Program (NMCP) (5, 20).

The current study reports the results of a longitudinal survey conducted in 2001, 2002 and 2005-2007 in the Binh Phuoc province of Vietnam. The study was undertaken to investigate the incidence of malaria caused by *P. falciparum* and to document the changes in genotype that were related to drug resistance. From this study we were able to identify the allelic and haplotype changes that occurred over the study years and deduce the factors associated with drug resistance.

MATERIALS AND METHODS

Study site and participants. This study was pre-approved by the Ethics and Scientific Committee of the NIMPE (Hanoi, Vietnam) and the Institute of Tropical Medicine, Nagasaki University (Nagasaki, Japan), and was performed in the Binh Phuoc provinces of southern Vietnam. The Study periods included June 2001, August and September 2002, May and October 2005, May and October 2006, and May and September 2007. We recruited 527 volunteers of the village residents in 2001, 687 in 2002, 1070 in 2005, 899 in 2006 and 634 in 2007. Information regarding age and gender were recorded for nearly all participants in 2001(507 of 527) and 2002 (682 of 687). This information was only recorded for positive participants in 2005, 2006 and 2007. Informed consent was obtained from all participants or their parents or guardians prior to their entry into the study. Blood samples were obtained from each participant and blood smears prepared for the identification of malaria infection microscopically. Blood samples collected onto filter paper were used for the genetic investigation of *P. falciparum*. Exclusion criteria for the analysis were pregnancy, splenectomy, severe malnutrition and reported prior treatment with antimalarial drug. Patients that demonstrated malaria infection microscopically were administered antimalarial drugs according to the Vietnamese Health policy.

PCR amplification and sequencing. DNA was extracted from the blood samples blotted onto filter paper according to the previously reported method (17). The primers used are presented in Table 1. Amplification was performed in 20 μl of reaction buffer containing 1 μl DNA, 0.5 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 2 mM $MgCl_2$ and 1 U *Taq* polymerase (Takara Bio, Inc., Ohtsu, Japan). For *pfcrt* and *pfmdr1,* outer PCR was performed under the following conditions: after an initial denaturation step at 94°C for 2 min, samples were subjected to 30 cycles of denaturation at 94°C for 20 s, hybridization at 52°C for 80 s, and DNA synthesis at 60°C for 4 min. The products of the outer PCR were then diluted 8-fold and used as a template for nested PCR using the following conditions: after an initial step of denaturation at 94°C for 2 min, samples were subjected to 35 cycles of denaturation at 94°C for 20 s, hybridization at 56°C for 80 s, and DNA synthesis at 60°C for 2 min. The PCR conditions for *pfdhfr* and *pfdhps* followed the method reported previously (11). The amplified products were then directly sequenced using the BigDye Terminator 1.1 cycle sequencing kit and a 3730 Genetic Analyzer (Applied Biosystems) (16). The primers used for sequence analysis are presented in Table 2. In case of new or rare mutations, two independent PCR products were subjected for sequence analysis. Additionally, sequence reactions were carried out from both 5' and 3'

sides.

Statistical analysis. Statistical analysis was performed using Stat View ver.5. The categorical variable was compared with the χ^2 test or the Fisher exact test. Continuous variables were analyzed using the *t*-test or the Spearman's rank correlation test. Odds ratios (OR) and 95% confidence intervals (CI) were computed as an estimate of the relative risk. Multiple logistic regression analysis was undertaken for variables associated with the year of the study, age, gender and genotypes related to drug resistance. $P<0.05$ was defined as statistically significant. Mixed haplotype infection cases were excluded from the haplotype statistical analysis. Correlations between genotype, age and gender were performed for the samples obtained between 2001 and 2002 only.

RESULTS

Characteristics of the study participants. The number of participants entered into this study was 527 in 2001, 687 in 2002, 1070 in 2005, 899 in 2006 and 634 in 2007. The rate of positive cases diagnosed microscopically was 24.5% (129), 24.0% (165), 3.4% (36), 5.0% (45) and 3.5% (22) for these years respectively (p=0.16). Among the participants, 52.5% (266 of 507) of the subjects investigated in 2001 were male, while 47.9% (327 of 682) were male in 2002. The average age was 24.9±17.9 (age range: 1-78) in 2001, and 23.6±17.1 (age range: 0-89) in 2002. Among the participants observed in 2001 and 2002, the positive rate for infection was 26.8% (159 of 593) for males and 22.5% (134 of 596) for females (p=0.08). The odds ratio for males was calculated at 1.26 with a 95% CI of 0.97-1.64. The average age of the participants negative for malaria infection in 2001 and 2002 was 25.4±17.9, and that of the positive participants was 20.5±14.6 (p<0.001). The gender proportion and average age of the positive participants for each year is presented in Table 3.

Genotype and time scale.

1) The *Pfcrt* Genotype. Substitution of K for T at codon 76, which encodes the critical change required for chloroquine resistance, was dominant in 2001 (T: 42/82, 51.2%), a

result that had rapidly decreased by 2005 (0%). In 2006 however, this genotype appeared to have reemerged, demonstrating a prevalence of 15.8% (T: 6/38), and then 18.8% (T: 3/16) in 2007. With regards to the haplotype present in codons 72-76, the mutant variations consisted of 2 types, CVIET and CVIDT. CVIET was observed to a high degree in 2001 (28/82, 34.1%), however its rate of observation was lower subsequent to 2002. CVIDT in 2001 shared 15.9% (13/82), and this tendency appeared stable, with the exception of 2005 (Figure 1).

Mutant alleles identified at positions 220 and 271 demonstrated a similar rate of occurrence, while the additional polymorphic loci showed different proportions (Table 3). The CVIET isolates exhibited 5 haplotype forms on the downstream side of position 76, in which the vast majority (79.4%, 27/34) were the same as Dd2 (CV**IET**-A-L-I-**S**-**E**-**S**-T-**T**-**I** at positions 72-76, 144, 148, 194, 220, 271, 326, 333, 356, 371). The remaining 4 haplotype forms with CVIET demonstrated the same substitution at positions 220, 271, 371, while the substitutions at 326, 333, 356 were found to be varied (N-T-I, N-**S**-I, N-S-**T**, N-**S**-**T** at positions 326, 333, 356). In contrast, the CVIDT isolates demonstrated 4 haplotype forms on the downstream part of position 76. The majority (90.9%, 30/33) were the same as the Cambodian isolate reported previously (4) (CV**IDT**-**F**-**I**-**T**-**S**-**E**-N-**S**-I-R at positions 72-76,

144, 148, 194, 220, 271, 326, 333, 356, 371). All other CVIDT variants demonstrated the same substitutions at position 220 and 271, while the substitutions at 144, 148, 194 and 333 demonstrated various combinations (A-L-**T**-T, A-L-**T-S**, **F-I**-S-**S** at positions 144, 148, 194, 333). Mixed infection cases were excluded from this haplotype statistical analysis.

2) Pfmdr1. Positions 86 N and 1246 D, sites that have previously been shown to exhibit variations in other studies, demonstrated no polymorphisms in the current study (23). The positions 130, 184, 1042 and 1109 exhibited polymorphisms, however the proportions identified over the study period were not statistically significant, irrespective of the uniform allelic pattern in 2005 (Table 3). The mutation on position 130 (E to K) has been reported recently in the Cambodian study sample (7).

***3) Pfdhfr* and *pfdhps*.** In relation to PfDHFR, all isolates showed normal patterns at positions 16 and 50, whose mutations are closely related to cycloguanil, pyrimethamine and trimethoprim resistance (18). The isolates demonstrating a lack of mutation were obtained in 2001 and 2002 only. All isolates obtained after 2005 possessed more than 2 mutations. While mutation at position 164, which is related to high level resistance, was found to demonstrate the highest percentage in 2001 (L: 28/74, 37.8%), a result that then tended to decrease over time (Table 3). PfDHPS mutation at position 437, which is regarded as both

the first and the most important change required for sulfadoxine and sulfamethoxazole resistance, showed a high proportion in every year. At position 436, 2 types of amino acid substitutions, S for A or F, were observed in this study.

Position 540 appeared to be more polymorphic in this study. We observed 3 types of substitutions, either K for E, N or Y. To the best of our knowledge, the substitution of K for Y has not been reported previously. The substitution of A for G at position 581 was observed every year in this study, and tended to increase in proportion from 12.7% (8/63) in 2001 to 52.9% (9/17) in 2007. The codon responsible for generating G is generally found to be ggg, however we identified ggt in some of the 2002 isolate samples. One of these was identified as a single infection, while two were found to result from a mixed infection and expressed gcg (A) or ggg (Table 3).

Related factors to the special genotypes. The positive samples 2001 and 2002 are used for the analysis. Among males and females, 36.3% and 42.9%, respectively, exhibited the *pfcrt*76T mutation (p=0.40). The average age of participants exhibiting the *pfcrt*76T mutation was significantly greater than that of the *pfcrt*76K mutation (25.7±15.0 vs 17.3±14.5, $p<0.01$) (Table 4). Using multivariate analysis for the risk with the isolates expressing *pfcrt*76T mutation, the year 2002 appeared to be accompanied with a lower risk

(p<0.01, 95%CI 0.13-0.52), while the ≥ 20 year old group demonstrated a greater risk (p=0.02, 95%CI 1.19-4.80). The number of mutations in the *pfdhfr* and *pfdhps* individually and combined were compared with age in infected participants using the Spearman's rank correlation test. The number of mutations were found to positively correlate with age for both genes and combined (*pfdhfr*; p=0.02, *pfdhps*; p=0.03, combined genes; p=0.02) (Table 5). Using multivariate analysis for ≥7 mutations, males demonstrated a reduced risk of disease infection (p=0.04, 95%CI 0.05-0.89), while an age of 20 years or older (p=0.05, 95%CI 1.01-16.59) and presence of the *pfcrt*76T mutation (p<0.01, 95%CI 2.92-59.32) appeared to represent independent risk factors. There were no significant associations between the study years (p=0.07, 95%CI 0.07-1.12).

The correlation between *pfcrt*76T and *pfmdr1* haplotypes was also calculated (Table 6). We found that 93.2% of the *pfcrt*76K isolates were wild type for *pfmdr1*, while 73.3% of the *pfcrt*76T isolates were wild type for *pfmdr1*. None of the *pfcrt*76K isolates demonstrated the double mutant haplotype (N**F**S**D**D) for *pfmdr1*, while 22.2% of the *pfcrt*76T isolates demonstrated the double mutant haplotype (N**F**S**D**D) for *pfmdr1* (p<0.001). The total number of mutations in *pfdhfr, pfdhps* and the combined genes were higher among the *pfcrt*76T isolates than the *pfcrt*76K isolates (p <0.001, 0.001, and <0.001,

respectively) (Table 6). Among the *pfmdr1* isolates with the double N**F**S**D**D mutations, the total number of mutations in the *pfdhfr* gene tended to be slightly higher (p=0.09) (Table 6).

DISCUSSION

Through the analysis of *P. falciparum* gene sequences in samples from malaria patients residing in southern Vietnam in 2001 and 2002, we observed a tendency for males to be more vulnerable than females to malaria infection, and that aging may be a protective factor. Although gender does not appear to be directly related to malaria vulnerability, except in pregnancy, socioeconomic situations may alter this tendency. In highly endemic areas, the risk of infection with malaria is greatest at 1-5 years of age. This risk gradually decreases as effective immunity develops. In the areas of moderate malaria transmission, a peak age of infection is observed later in childhood. In low transmission areas, vulnerability to infection does not appear to vary among the different ages, as immunity is not long lasting (2). In this study, the transmission level in 2001 and 2002 was moderate, so immunity accompanied with aging may partially work in preventing malaria infection.

The number of isolates with the *pfcrt*76T mutation was found to rapidly decrease between 2001 and 2005. We hypothesized that this was the result of a reduction in drug pressure of chloroquine due to the preferred use of artemisinin derivatives in clinical settings. In Malawi, the efficacy of chloroquine in the treatment of *P. falciparum* malaria was calculated to be less than 50% in 1993, however after the replacement of chloroquine

treatment in 2001, the efficacy was increased to 99% (9). An additional analysis also demonstrated that the chloroquine resistant mutation *pfcrt*76T was detected in 85% of isolates in 1992, which was reduced to 13% in 2000 (8). We did not detect parasites expressing the *pfcrt*76T in 2005, however the mutant isolates were found to have reemerged in 2006 and 2007. One possible explanation for this finding may be the existance of lasting chloroquine pressure, as the *P. vivax* remains endemic in this area and thus chloroquine is still prescribed.

The variation of haplotypes observed in *pfcrt* may be explained in part by the combination of the parental types CVIET Dd2 and the reported CVIDT Cambodia type. These findings suggest that recombination of *pfcrt*, including regions within exons, occur frequently and that these recombinations produce animate descendents. It remains unclear whether these variants demonstrate a similar infectious ability to their parental variants. Further investigations *in vitro* and *in vivo* may be useful in clarifying this situation. In addition, analysis using several microsatellite markers located both inside and around the *pfcrt* locus may aid in the definition of genetic diversity (3, 13).

In the Cambodian study conducted between 2001 and 2002, Khim et al. reported variations in *pfmdr1* haplotypes that were similar to those reported in this study. They

observed mutations located at positions 130 and 1109 for the first time (7). We also identified these mutations in our samples. Since the site of the Cambodian study was within close proximity to our study site, these isolates may have been generated by a low level clonal spread across these areas. In Vietnam, Ngo et al. investigated the *pfmdr1* genotype in Binh Phuoc province and detected 3 patterns of *pfmdr1* haplotypes (N-S-N-D, **Y**-S-N-D and N-S-**D**-D at positions 86-1034-1042-1242) (14).

We also identified polymorphisms in the *Pfdhfr* gene at positions 51, 59, 108 and 164, but not at positions 16 or 50. The number of substitutions observed is known to correlate with the level of pyrimethamine resistance. In our study, isolates with 4 mutations strains were prevalent in 2001 (44.4%), however the number was found to rapidly reduce**.** In addition, the isolates without mutations also tended to decrease in rate.

Substitutions at the 5 loci of the *pfdhps* gene including S436A/F, A437G, K540E/N, A581G and A613T/S have been implicated in the development of resistance by decreasing the binding affinity of the enzyme (23). In this study, we observed all of these mutations and identified 1 novel type of Y mutation on position 540. The substitution K540N has been reported recently on the Car Nicobar Island in India. The parasite harboring this mutation was isolated in 2005, and the authors indicated that the mutation was caused by

additional selective pressure of cotrimoxazole (sulfamethoxazole-trimethoprim) with SP following the 2004 Tsunami. Computer modeling and docking analysis of K540N also demonstrated that the binding affinity of PfDHPS with K540N was similar to that of K540E (10).

Although the substitution of 581A for G was originally reported in South America (23), it is now also widespread among Asian countries. The report from Cambodia documented that 65.5% of the total isolates also contained this mutation (7). In Vietnam, a few isolates expressing this amino acid substitution have been reported. The study by Masimirembwa (12), Phuc (15) and coworkers independently demonstrated that 1 in 40 and 3 in 40 respectively, expressed these mutations. The substitutions reported are encoded by the ggg codon and a codon that we newly found was synonymously changed with ggt.

Wargo et al. demonstrated that antifolate resistant parasites were competitively suppressed by sensitive parasites in the absence of drug pressure using a rodent *Plasmodium chabaudi* model (22). This finding suggests the possibility that lasting antifolate drug pressure in this area due to either the prescription of SP or trimethoprim contained in CV8, or from the prescription of cotrimoxazole as an antibiotic for bacterial infectious disease and anti-pneumocystis infection in HIV patients. The rapid spread of

cotrimoxazole resistant *Streptococcus pneumoniae* in Vietnam has been presented in the Asian Network for Surveillance of Resistant Pathogens (ANSORP) study. This report documented that in Vietnam the rate of cotrimoxazole non-susceptible *S. Pneumoniae* was as high as 91.3%. This high rate indicated high pressure of cotrimoxazole in Vietnam (19).

Interestingly, we also demonstrated a weak correlation between female gender and two genetic makers of drug resistance, *pfcrt*76T and a high number of mutations in both *pfdhfr* and *pfdhps* combined. Using multivariate analysis, *pfcrt*76T did not appear to demonstrate a statistical difference in correlation with female gender, while the higher antifolate resistant genotype did. In addition, we observed a strong correlation between these genetic makers and aging. Regarding the results that the patients in the older age groups demonstrated a reduced vulnerability to malaria infection in this study, we can make hypothesis that females and older people may prefer to take antimalarial or antibiotic medications which might cause low infectious rate among these people.

In this study, the isolates with *pfcrt*76T demonstrated a greater number of mutations in *pfdhfr* and *pfdhps*. A similar result was reported in a study undertaken in Sudan, where a correlation between *pfcrt*72-76 CVIET and 4 mutations in *pfdhfr* and *pfdhps* was observed (6). The authors suggested that administration of sulfadoxine-pyrimethamine in the

treatment of infections harboring chloroquine resistant isolates induces *pfdhfr/pfdhps* mutations. The situation in Vietnam however may prove to differ and thus further investigation using microsatellite marker analysis may be required to define genetic movement. Such findings may aid in the development of strategies for the eradication of malaria from endemic sites.

Acknowledgements:

The authors are grateful to all the participants and the staff members of Local Binh Phuoc Clinics for their kind assistance. We thank Professor K. Tanabe and Dr. N. Sakihama of Osaka University for their advice on blood collection and parasite DNA isolation.

This work was supported by a Grant-in-Aid for Scientific Research (KAKENHI, 19406011 to H.U.) from the Japan Society for Promotion of Science, and a Core University Program of Japan Society for Promotion of Science, the Collaborative Study on Emerging and Re-emerging Infectious Diseases in Vietnam.

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Figure Legend:

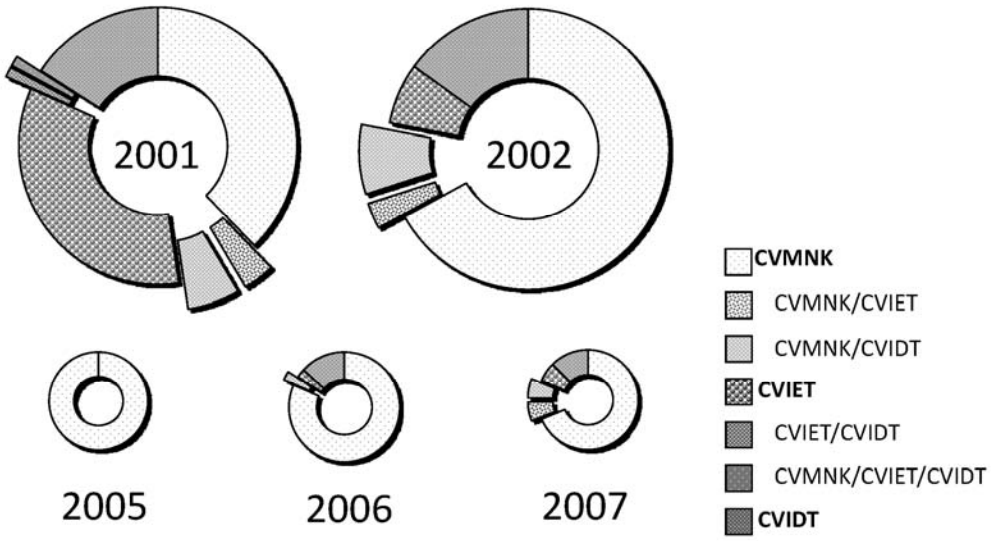

Fig.1. Sequence polymorphism for *pfcrt* at positions 72-76. Each pie graph represents a sample year. The square of the circles reflects positive ratio each year.

Note: the mixed infections are presented at the pop-up positions.

Table 1. Primers used to genotype *pfcrt, pfmdr1, pfdhfr* and *pfdhps*.

Primer	Sequence (5'→3')	Purpose
Crt1F	cattgtcttccacatatatgacataaa	Outer PCR for *pfcrt*
Crt4R	gatctctataccttcaacattattcct	Outer PCR for *pfcrt*
Crt2F	tttcccttgtcgaccttaacagatggc	Nested PCR for former part of *pfcrt*
Crt12R	atcctattttacctctacgactgt	Nested PCR for former part of *pfcrt*
Crt11F	tttcttataggctatggtatccttt	Nested PCR for latter part of *pfcrt*
Crt15R	ttttaatttcacacttaccaaagt	Nested PCR for latter part of *pfcrt*
MDR2F	aaagatggtaacctcagtatcaaagaaga	Outer and nested PCR for former part of *pfmdr1*
MDR8R	atgattcgataaattcatctatagcagcaa	Outer and nested PCR for former part of *pfmdr1*
MDR10R	ttttttggacacatcaacaacatcagaatc	Nested PCR for former part of *pfmdr1*
MDR5F	agaagattatttctgtaatttgatagaaaaagc	Nested PCR for former part of *pfmdr1*
DHFR1F	atgatggaacaagtctgcgacgttttcgat	PCR for *pfdhfr*
DHFR2R	ttcatttaacattttattattcgttttctt	PCR for *pfdhfr*
DHPS1F	ccattcctcatgtgtatacaacac	PCR for *pfdhps*
DHPS2R	gttttaatcacatgtttgcactttc	PCR for *pfdhps*

Table 2. Primers for sequencing

Primer	Sequence (5'→3')	Target gene	Objective loci
CRT2.2F	ttcgaccttaacagatggctcacgttta	*pfcrt*	72-76, 97
CRT9.2F	tatttcttatgacctttttaggaacga	*pfcrt*	144, 148, 160, 163
CRT10.2F	atttatttactccttttttagatatcac	*pfcrt*	194, 220
CRT11.2F	attcctataacgcattataattatttc	*pfcrt*	271
CRT16R	aaacattcccatatttatttcctc	*pfcrt*	326, 333
CRT14F	ttatagattatcgacaaattttct	*pfcrt*	356
CRT17F	gtacaacgtatcatattttataat	*pfcrt*	371
MDRp3F	gagtaccgctgaattatttaga	*pfmdr1*	86, 147,153, 184
MDR14F	atgtttatgttaacaattatcttacca	*pfmdr1*	363, 487
MDR7R	gcacattaattttccagcataactaccagt	*pfmdr1*	1033, 1034
MDR6F	agaattattgtaaatgcagctttatggggattc	*pfmdr1*	1042, 1109
MDR 15F	atgatcacattatattaaaaaatgat	*pfmdr1*	1246
DHFR2R	ttcatttaacattttattattcgttttctt	*pfdhfr*	16, 50, 51, 59, 108, 140, 164
DHPS3F	tttgttgaacctaaacgtgctgttcaaa	*pfdhps*	436, 437
DHPS4R	tttattttcattttgttgttcatcatg	*pfdhps*	540, 581, 613

Table 3. Characteristics of participants and parasite genotypes.

	Position	Amino acid	2001	2002	2005	2006	2007	P
Age*			22.1±15.2 (2-	19.3±14.0 (0-72)	23.1±16.3 (1-	14.3±11.8 (0-	9.63±10.8 (2-	0.05
Gender		52.7	55.5	66.7	38.1	25.0	0.07	
pfcrt	74	M/**I**/mix	31/42/9 (37.8/51.2/11.0)	67/22/11 (67.0/22.0/11.0)	33/0/0 (100/0/0)	31/6/1 (81.6/15.8/2.6)	11/3/2 (68.8/18.8/12.5)	<0.001
	75	N/**E**/**D**/mix	31/28/13/10 (37.8/34.1/15.9/12	67/7/15/11 (67.0/7.0/15.0/11.0)	33/0/0/0 (100/0/0/0)	31/1/5/1 (83.8/2.6/13.2/2.6	11/1/2/2 (68.8/6.3/12.5/12.	<0.001
	76	K/**T**/mix	31/42/9 (37.8/51.2/11.0)	67/22/11 (67.0/22.0/11.0)	33/0/0 (100/0/0)	31/6/1 (81.6/15.8/2.6)	11/3/2 (68.8/18.8/12.5)	<0.001
	144	A/**F**/mix	63/12/7 (76.8/14.6/8.5)	75/17/8 (75.0/17.0/ 8.0)	36/0/0/ (100/0/0)	35/3/0 (92.1/7.9/0)	14/2/0 (87.5/12.5/0)	0.07
	148	L/**I**/mix	63/12/7 (76.8/14.6/8.5)	75/17/8 (75.0/17.0/ 8.0)	36/0/0 (100/0/0)	35/3/0 (92.1/7.9/0)	14/2/0 (87.5/12.5/0	0.07
	194	I/**T**/mix	63/12/7 (76.8/14.6/8.5)	75/17/8 (75.0/17.0/ 8.0)	36/0/0 (100/0/0)	35/3/0 (92.1/7.9/0)	14/2/0 (87.5/12.5/0)	0.07
	220	A/**S**/mix	31/42/9 (37.8/51.2/11.0)	65/24/1 (65.0/24.0/11.0)	36/0/0 (100/0/0)	31/6/1 (81.6/15.8/2.6)	11/4/1 (68.8/25.0/6.3)	<0.001
	271	Q/**E**/mix	31/42/9 (37.8/51.2/11.0)	68/22/10 (68.0/22.0/10.0)	36/0/0 (100/0/0)	31/5/2 (81.6/13.2/5.3)	11/4/1 (68.8/25.0/6.3)	<0.001
	326	N/**S**/mix	54/23/6 (65.9/28.0/7.3)	93/3/4 (93.0/3.0/4.0)	36/0/0 (100/0/0)	36/2/0 (94.7/5.3/0)	15/0/1 (92.1/0/6.3)	<0.001
	333	T/**S**/mix	59/17/7 (72.0/20.7/8.5)	76/16/8 (76.0/16.0/8.0)	36/0/0 (100/0/0)	35/3/0 (92.1/7.9/0)	13/2/1 (81.3/12.5/6.3)	0.02
	356	I/**T**/mix	55/24/4 (67.1/29.3/4.9)	90/6/4 (90.0/6.0/4.0)	36/0/0 (100/0/0)	36/2/0 (92.1/5.3/0)	15/0/1 (93.8/0/6.3)	<0.001
	371	R/**I**/mix	49/28/6 (59.8/34.1/7.3)	89/7/4 (89.0/7.0/4.0)	36/0/0 (100/0/0)	35/1/2 (92.1/2.6/5.3)	13/1/2 (81.3/6.3/12.5)	<0.001
pfmdr1	130	E/**K**	66/1 (98.5/1.5)	90/1 (98.9/1.1)	33/0 (100/0)	35/0 (100/0)	13/1 (92.9/7.1)	-
	184	Y/**F**/ mix	53/8/5 (80.3/12.7 / 7.9)	72/7/12 (79.1/7.7 / 13.2)	33/0/0 (100/0/ 0)	31/3/1 (88.6/8.6/ 2.9)	13/1/0 (92.9/7.1/ 0)	0.32
	1042	N/**D**/mix	55/8/4 (83.3/12.1/ 6.0)	80/4/7 (87.9/4.4/7.7)	33/0/0 (100/0/ 0)	33/3/0 (91.7/8.3/ 0)	12/1/1 (85.7/7.1/ 7.1)	0.17
	1109	V/**I**/mix	62/0/4 (93.9/0/ 6.1)	91/1/0 (98.9/1.1/ 0)	33/0/0 (100/0/ 0)	33/3/0 (91.7/8.3/ 0)	13/0/0 (100/0/ 0)	-
pfdhfr	51	N/**I**/mix	4/71/0 (5.3/94.7/ 0)	6/39/5 (12.0/78.0/ 10.0)	1/23/0 (4.2/95.8/ 0)	10/24/0 (29.4/70.6/ 0)	3/8/0 (27.3/72.7/ 0)	<0.001
	59	C/**R**/mix	4/70/1 (5.3/93.3/1.3)	3/43/4 (6.0/86.0/8.0)	0/22/2 (0/91.7/8.3)	0/34/0 (0/100/ 0)	0/10/0 (0/100/ 0)	0.39
	108	S/**N**/mix	4/71/0 (5.3/94.7/0)	1/48/1 (2.0/96.0/ 2.0)	0/24/0 (0/100/ 0)	0/35/0 (0/100/ 0)	0/11/0 (0/100/ 0)	0.38
	164	I/**L**/mix	36/28/10 (48.6/37.8/ 13.5)	40/5/5 (80.0/10.0/ 10.0)	21/3/0 (87.5/12.5/ 0)	32/3/0 (91.4/8.6/ 0)	9/2/1 (75.0/16.7/ 8.3)	<0.001
pfdhps	436	S/**A**/**F**/mix	21/25/10/7 (33.3/39.7/15.9/11	44/33/8/10 (46.3/34.7/8.4/10.7)	10/19/1/0 (33.3/63.3/3.3/0)	25/3/0/8 (69.4/8.3/0/ 22.3)	13/0/1/2 (81.3/0/6.3/12.5)	<0.001
	437	A/**G**/mix	7/56/0 (11.1/88.9/ 0)	0/92/3 (0/96.8/ 3.2)	1/29/0 (3.3/96.7/ 0)	1/34/1 (2.8/94.4/ 2.8)	0/17/0 (0/100/ 0)	<0.001
	540	K/**E**/**N**/**Y**/	19/27/6/0/11 (30.2/63.5/9.5/0/1	32/39/7/1/17 (33.3/40.6/7.3/1.0/1	7/19/1/0/3 (23.3/63.3/3.3/0/1	6/19/0/0/12 (16.2/51.4/0/0/32.	1/9/0/0/7 (5.9/52.9/0/0/41.2	0.23
	581	A/**G**/mix	47/8/8 (74.6/12.7/12.7)	61/20/16 (62.9/19.6/17.4)	23/4/3 (76.7/13.3/10.0)	8/16/13 (21.6/43.2/35.1)	4/9/4 (23.5/52.9/23.5)	<0.001
	613	A/T/S/mix	49/1/8/5 (77.8/1.6/12.7/8.0)	87/0/6/3 (90.6/0/6.3/3.1)	30/0/0/0 (100/0/0/0)	36/0/0/0 (100/0/0/0)	15/0/0/0 (100/0/0/0)	<0.001

1. NOTE. Bold type indicates amino acid mutations. The relative % of the genotype is presented in parentheses.

2. * Mean ± S.D (minimum-maximum).

3. ** Correlation between each year. Mix infections of each locus were excluded from the statistical analysis.

Table 4. Correlation between genotype and patient characteristics.

		Gender					
		No. (%) of Male	No. (%) of Female	P value	Age*	No. of people	P value
pfcrt	76K	58 (63.7)	40 (57.1)		17.3±14.5	98	
	76**T**	33 (36.3)	30 (42.9)	0.4	25.7±15.0	63	<.01
*pfmdr1***	NYSND	63 (86.3)	55 (88.7)		19.0±15.1	118	
	N**F**SND	1 (1.4)	4 (6.5)		10.6± 6.5	5	
	NYS**D**D	2 (2.7)	0 (0)		22.5± 7.8	2	
	N**F**S**D**D	7 (9.6)	3 (4.8)	0.16	22.5±14.5	10	0.1
Pfdhfr + *pfdhps*†	≥5	31 (79.5)	27 (81.8)	0.8			
	≥6	18 (46.2)	20 (60.0)	0.22			
	≥7	11 (28.2)	14 (42.4)	0.21			

NOTE Excluding mixed infections. n= 91 for males, n= 70 for females. Bold type indicates amino acid mutations.

*mean ± S.D

***pfmdr1* position 86_184_1034_1042_1246.

†The mutation number for *pfdhfr* and *pfdhps*.

Table 5. The mutation numbers for *pfdhfr*, *pfdhps* and age of patients.

Gene	No. of mutations	Age*	No. of people	P value
pfdhfr	0	24.6±12.4	8	
	2	19.5±24.7	2	
	3	17.5±15.9	61	
	4	27.8±14.2	31	0.02
pfdhps	0	25.4± 9.1	5	
	1	18.0±18.1	10	
	2	17.9±15.6	36	
	3	21.1±13.4	50	
	4	30.3±15.6	14	0.03
Pfdhfr + *pfdhps*	0	27.0± 9.6	4	
	2	16.3±13.2	3	
	3	10.5±12.0	2	
	4	18.3±21.7	4	
	5	19.1±15.5	21	
	6	17.6±12.4	13	
	7	26.7±13.8	19	
	8	32.8±12.3	6	0.02

*Mean ± S.D

Table 6. Relationship between different genotypes.

		*pfcrt*76			*pfdhfr*			*pfdhps*					
		No. (%) of K	No. (%) of **T**	P value	A	No. of samples	P value	B	No. of samples	P value	A+B	No. of samples	P value
*pfcrt*76	K				2.7±1.1	51		2.2±1.1	60		4.6±2.0	35	
	T				3.4±1.0	43	<0.001	2.8±0.7	46	<0.001	6.3±1.5	34	<0.001
pfmdr1	NYSND*	69 (93.2)**	33 (73.3)**		3.1±0.9	66		2.6±0.9	80		4.0±2.0	49	
	N**F**SND*	4 (5.4)**	1 (2.2)**		2.0±1.7	3		2.3±1.0	4		5.6±1.6	3	
	NYS**D**D*	1 (1.4)**	1 (2.2)**		3	1			0			0	
	N**F**S**D**D*	0 (0)**	10 (22.2)**	<.001	3.6±0.5	8	0.09	2.5±1.0	6		6.0±1.5	6	

NOTE Bold type indicates amino acid mutations.

A: represents the average mutation number ± SD in *pfdhfr* among each genotype group for *pfcrt* or *pfmdr1*, and B represents that in *pfdhps*.

**pfmdr1* position 86_184_1034_1042_1246. **Proportion (%) of the *pfmdr1* genotypes among *pfcrt* 76K or 76T.