IN VITRO SUSCEPTIBILITY OF *PLASMODIUM FALCIPARUM* ISOLATES TO CHLOROQUINE AND MEFLOQUINE IN SOUTHEASTERN MINDANAO ISLAND, THE PHILIPPINES

Toshimitsu Hatabu[1,2], Shin-ichiro Kawazu[1], Jun Suzuki[3], Reuben F Valenzuela[4], Elena A Villacorte[4], Mamoru Suzuki[5], Pilarita T Rivera[4] and Shigeyuki Kano[1]

[1]Research Institute, International Medical Center of Japan, Tokyo; [2]Gunma University School of Health Sciences, Gunma; [3]Department of Microbiology, The Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan; [4]Department of Parasitology, College of Public Health, University of the Philippines-Manila, Manila, The Philippines; [5]Department of Parasitology, Gunma University Graduate School of Medical Sciences, Gunma, Japan

Abstract. Although the presence of multi-drug-resistant falciparum malaria has been reported in the Philippines, the distribution of drug-resistant malaria parasites has not yet been determined in Mindanao Island. *In vitro* susceptibility of *P. falciparum* to both chloroquine and mefloquine was assessed to forecast the spread of drug-resistant parasites in various foci in southeastern Mindanao Island. Of the 33 isolates of *P. falciparum* successfully tested, 10 (30%) were susceptible, 12 (36%) showed decreased susceptibility (80 nM ≤ IC_{50} < 114 nM), and 11 (33%) were resistant (IC_{50} ≥ 114 nM) to chloroquine. Ten (91%) of the resistant isolates and 9 (75%) of those with decreased susceptibility were from northern and northwestern Davao del Norte Province. Chloroquine-susceptible isolates were found among patients in the eastern parts of Davao del Norte and Davao Oriental provinces. Seven isolates from several foci in the study area were all mefloquine- susceptible (IC_{50} < 10 nM). This is the first report indicating the potential emergence of chloroquine-resistant *P. falciparum* on Mindanao Island, which is presently regarded as a drug-susceptible area.

INTRODUCTION

Malaria is one of the major fatal parasitic diseases endemic to many tropical and subtropical regions of the world. Global distribution of drug-resistant *Plasmodium falciparum* is making malaria treatment increasingly difficult (White, 1996; Alrajhi *et al*, 1999). Chloroquine had been the most reliable antimalarial drug for more than three decades until the emergence and spread of drug-resistant *P. falciparum* rendered its application ineffective in much of the world (White, 1996; Alrajhi *et al*, 1999). However, chloroquine remains the drug of choice in a number of malaria-endemic foci for the treatment of uncomplicated malaria (Okonkwo *et al*, 2001; Thanh *et al*, 2001).

Correspondence: Shigeyuki Kano, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan.
Tel: +81-3-3202-7181 (ext. 2877); Fax: +81-3-3202-7364
E-mail: kano@ri.imcj.go.jp

In the Philippines, malaria is a major public health problem; about 11 million people live in endemic areas. Approximately 31% of the population lives in high malaria transmission areas (Pilarita *et al*, 1999). Shute *et al* (1972) first reported *in vitro* amodiaquine resistance of *P. falciparum* isolates in the Philippines. As of 1996, between 23 to 39% of all cases of falciparum malaria in Palawan Island were reported to be resistant to chloroquine (Baird *et al*, 1996). Although the existence of chloroquine-resistant parasites in the northeastern Philippines has been reported, local distribution of drug-resistant isolates on Mindanao Island has not yet been evaluated. Because the island is close to Indonesia, where drug-resistant malaria parasites are very prevalent (Verdrager *et al*, 1976; Pribadi *et al*, 1992), it is important to survey the drug susceptibility of isolates in the Mindanao region. In this study, the *in vitro* susceptibility of *P. falciparum* isolates to chloroquine and mefloquine was assessed to clarify the current distribution of drug-resistant falciparum malaria on Mindanao Island.

MATERIALS AND METHODS

Study area

This study was conducted at Davao Regional Hospital in Tagum City, Davao del Norte Province, located in southeastern Mindanao Island (Fig 1), between August 1999 and March 2001. In this province, malaria transmission occurs throughout the year, with two peaks, one in July/September and the other in December/January. *Plasmodium falciparum* is the predominant parasite species, whereas the frequency of *P. vivax* is about 30%. All areas of Davao del Norte Province, except Tagum City, are known to be highly endemic for malaria, with the incidence of cases ranging from 0.2 to 47.9 per 1,000 population in 1993 (Pilarita *et al*, 1999). Chloroquine is currently the drug of choice for the treatment of uncomplicated malaria. Most hospitalized patients come from Asuncion, Kapalong, Laak, and Compostella in Davao del Norte Province, or from the nearby provinces of Agusan del Sur and Davao Oriental (Fig 1).

Patients and sampling strategy

Fifty-seven isolates of *P. falciparum* were obtained from symptomatic Filipino patients admitted to Davao Regional Hospital. Giemsa-stained thin blood smears were examined for identification of *Plasmodium* species. After informed consent was obtained from the patients, venous blood was collected in a tube coated with EDTA (Venoject vacuum tube, Terumo, Tokyo, Japan). All samples were drawn before any medication was given. Part of each blood sample (2 ml) was mixed with an equal volume of stock solution (glycerol 35 g, D-sorbitol 3.0 g, NaCl 0.65 g in 100 ml distilled water) for freezing and was preserved in a dry, liquid nitrogen shipping container (Vapor Shipper, MVE Inc, New Prague, MN) for transport to Tokyo, where the samples were stored in liquid nitrogen until use.

In vitro drug susceptibility test

Thirty-three of the 57 *P. falciparum* isolates were successfully cultivated and used for *in vitro* drug susceptibility testing. The *in vitro* drug susceptibility test used in this study was a modified semi-microtest described previously (Inaba *et al*, 2001). The cryopreserved blood sample was cultured by the standard method using a multi-gas incubator until parasitemia reached at least 1%. The parasites were then synchronized with 5% D-sorbitol for 15 minutes at room temperature and were washed with RPMI 1640 medium (Gibco BRL, Rockville, MD) 3 times by centrifugation at 400*g* for 5 minutes. After washing, the erythrocytes were resuspended in RPMI 1640

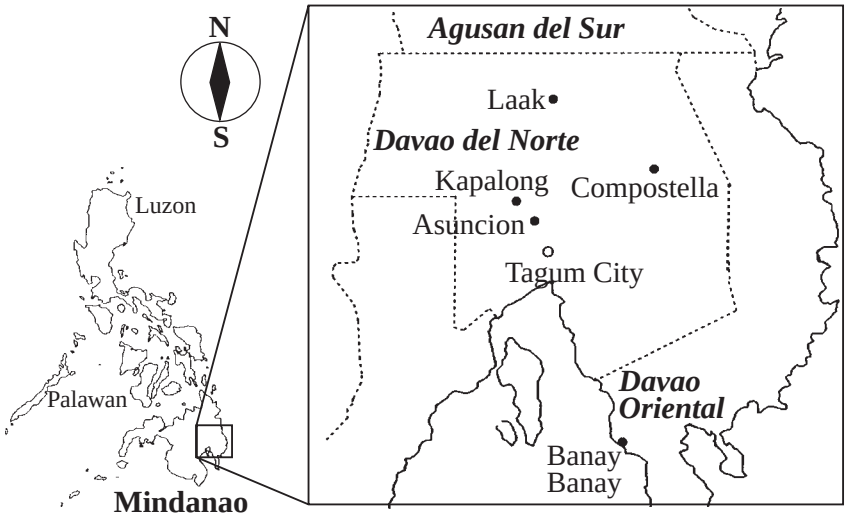

Fig 1–Location of Davao del Norte Province on Mindanao Island and the locations of the cities where the survey was conducted.

medium supplemented with 10% human serum (from non-immune Japanese donors without a previous history of malaria), 25 mM HEPES, 25 μg ml^{-1} gentamicin, and sodium bicarbonate at a hematocrit of 5% and initial parasitemia of 1-5%. Five hundred microliters of the erythrocyte suspension were placed in each well of a tissue culture plate (24-well flat bottom, Corning Costar, New York, NY). Twenty microliters of chloroquine diphosphate were added to each well to give a series of doubling dilutions from 20 to 10,240 nM. To monitor parasite growth, six wells per plate served as controls without chloroquine. After 24 hours' incubation, the control wells were monitored for parasite growth by observing Giemsa-stained thin-smeared specimens. If the trophozoites had not matured to schizonts, the control wells were checked every 6 hours thereafter. When the schizonts were observed to be fully grown in the control wells, the culture plate was removed from the incubator. Thin-smear specimens stained with Giemsa solution were made from the contents of each well, and the numbers of erythrocytes counted under a microscope in the control smears until 50 schizonts were encountered. The effect of chloroquine on parasite growth was evaluated by observing the decreased numbers of schizonts per equal numbers of erythrocytes counted previously in the control cultures. The growth inhibition effect (%) was calculated as follows: test well schizont count/control well schizont count (50) x 100.

Data analysis

The drug concentration inhibiting parasite growth by 50% (IC_{50}) was calculated by the probit method (Inaba *et al*, 2001; de Franciso *et al*, 1988). Isolates with IC_{50} values between 80 and 114 nM were considered to have decreased susceptibility, and those with $IC_{50} \geq 114$ nM were regarded as resistant (Inaba *et al*, 2001). The threshold of the IC_{50} value for mefloquine resistance was considered to be 40 nM (Milijaona *et al*, 2000). The Student's *t*-test or Welch's *t*-test were used for statistical analysis. A p-value < 0.05 was considered statistically significant.

RESULTS

The clinical characteristics of the 33 malaria

Table I
Characteristics of study patients grouped by city of origin.

	Asuncion	Kapalong	Laak (Davao del Norte Province)	Compostella	Agusan (Agusan del Sur Povince)	Banay Banay (Davao Oriental Province)
No. of patients	10	5	8	3	4	3
Male/female	7/3	1/4	5/3	3/0	2/2	2/1
Age (years)	24.4±11.8	37.4±21.5	18.8±11.5	22.9±7.6	21.8±10.6	36.7±10.2
Body temp (ºC)	38.6±0.82	37 (n=2)	38.04±1.25 (n=5)	37.4±1.38	39.2±0.25	37.6±0.96
Hgb (g/l)	96.65±19.64	95.5 (n=2)	89.93±33.33 (n=4)	91.13±30.58	99.13±13.88	105.9±15.4
Parasite count (/μl)	49,618.4±26,10.7	33,700 (n=l)	40,363.3±33,327.3	448,799.7±565,000.2	14,300.0±2,066.7	17,700 (n=2)

Mean±SD data are shown.

Table 2
Susceptibility of Mindanao isolates of *P. falciparum* to chloroquine, by province.

	Davao del Norte	Agusan del Sur	Davao Oriental
No. of isolates tested	2	6 4	3
Susceptible (IC_{50} <80 nM)	6 (23.0)	1 (25.0)	3 (100)
Decreased susceptibility	10 (38.5)	2 (50.0)	0
Resistant(IC_{50} ≥ 114 nM)	10 (38.5)	1 (25.0)	0
With IC_{100} ≥ 640 nM	17 (65.4)	2 (50.0)	1 (33.3)
Minimum IC_{50} (nM)	37	27	43
Maximum IC_{50} (nM)	263	187	75
Mean IC_{50}±SD (nM)	110 ± 54	104 ± 42	64 ± 14

Mean±SD data are shown.
Percentages are shown in parentheses.

patients on admission are summarized in Table 1. There were no significant differences in age, sex, body temperature, or hemoglobin concentration between the groups of patients from different locales. The results of the *in vitro* chloroquine susceptibility tests are shown in Table 2. Twenty-six of the 33 isolates of *P. falciparum* originated from Davao del Norte, 4 isolates were from Agusan del Sur, and 3 were from Davao Oriental. Of the 33 isolates, 11 (33%) were resistant to chloroquine, 12 (36%) had decreased susceptibility, and 10 (30%) were found to be susceptible. The IC_{50} values for chloroquine varied from 27-263 nM, with a geometric mean (±SD) of 104 (±53) nM. Ten of the 11 (91%) resistant isolates and 10 of the 12 (83%) isolates with decreased susceptibility were from Davao del Norte Province. One of the 11 resistant isolates and 2 of the 12 isolates with decreased susceptibility were from Agusan del Sur Province. All the isolates from Davao Oriental Province were considered chloroquine-susceptible. The geometric mean (±SD) of IC_{50} values [110 (±54) nM] recorded for the isolates from Davao del Norte Province was higher than that recorded for the isolates from Agusan del Sur Province [104 (±42) nM], but the difference was not significant. The geometric mean (±SD) of IC_{50} values for the isolates from Davao Oriental Province [64 (±14) nM] was significantly lower than that for the isolates from Davao del Norte Province (p=0.006).

Detailed results of *in vitro* chloroquine susceptibility testing in *P. falciparum* isolates from towns in Davao del Norte Province are shown in Table 3. The highest IC_{50} (263 nM) to chloroquine was recorded for one isolate from Asuncion, which is located in southern Davao del Norte Province. Of ten isolates from Asuncion, six (60%) were resistant and three (30%) had decreased sensitivity to chloroquine. The IC_{50} values of these isolates to chloroquine varied from 55-263 nM, with a geometric mean (±SD) of 133 (±41) nM. Isolates from Kapalong, located in southern Davao del Norte Province close to Asuncion, showed IC_{50} values in the range 51-129 nM. The geometric mean (±SD) of the IC_{50} values of the Kapalong isolates (82 (±25) nM) were significantly lower than the isolates from Asuncion (p = 0.025). Eight isolates from Laak, which is located in northern Davao del Norte Province, had IC_{50} values of 41-224 nM, with a geometric mean (±SD) of 108 (±36) nM. Differences between the mean IC_{50} values of isolates from Laak and those from Asuncion were not significantly different. Three isolates from Compostella, which is located in eastern Davao del Norte Province, had IC_{50} values of 37-104 nM. The geometric mean (±SD) of the IC_{50} value of the Compostella isolates [75 (±25) nM] was significantly lower than the Asuncion isolates (p = 0.043). The seven isolates from Asuncion, Laak, and the provinces of Agusan del Sur and Davao Oriental were successfully tested for susceptibility to mefloquine and all proved highly susceptible to mefloquine (IC_{50} values < 10 nM) (data not shown).

Table 3
Susceptibility of Davao del Norte isolates of *P. falciparum* to chloroquine by city of origin.

	Asuncion	Kapalong	Laak	Compostella
No. of isolates tested	10	5	8	3
Susceptible (IC_{50} < 80 nM)	1 (10.0)	2 (40.0)	2 (25.0)	1 (33.3)
Decreased susceptibility	3 (30.0)	2 (40.0)	3 (37.5)	2 (66.7)
Resistant (IC_{50}≥114 nM)	6 (60.0)	1 (20.0)	3 (37.5)	0
With IC_{100} ≥640 nM	7 (70.0)	2 (40.0)	8 (100)	0
Minimum IC_{50} (nM)	55	51	41	37
Maximum IC_{50} (nM)	263	129	224	104
Mean IC_{50}±SD (nM)	133 ± 41	82 ± 25[a]	108 ± 36	75 ± 25[a]

Mean±SD data are shown; [a]p< 0.05 compaired with isolates from Asuncion.

DISCUSSION

To call attention to the emergence and spread of drug-resistant malaria in the Philippines, the current state of drug susceptibility of *P. falciparum* isolates on Mindanao Island was investigated. The results indicated that two groups of parasites with different susceptibility to chloroquine were distributed in Davao del Norte Province. Three genes, *pfmdr1, cg2*, and *pfcrt* have been reported to be responsible for resistance of *P. falciparum* to chloroquine in other parts of the world (Adagu *et al*, 1999; Babiker *et al*, 2001; Wellems *et al*, 2001). The varieties of chloroquine susceptibility seen in Davao del Norte isolates may be attributed to differences in mutations of one or more of these genes. The genetic backgrounds of the isolates should be further investigated to determine how parasites with various degrees of chloroquine resistance are spreading in this province.

Distribution of chloroquine-resistant malaria parasites in Davao del Norte Province was regionally limited, and the levels of parasite drug-resistance were generally low. Le Bras *et al* (1984) reported that the median IC_{50} value for chloroquine obtained by *in vitro* testing was less than 120 nM in 22 isolates from patients who had been successfully treated with chloroquine, whereas the IC_{50} was more than 250 nM in isolates from 6 patients with clinically chloroquine-resistant malaria. Based on these results, the *P. falciparum* isolates categorized as chloroquine-resistant in the present study by *in vitro* testing may still be sensitive to chloroquine *in vivo*. Counter-measures should be taken before chloroquine becomes ineffective on Mindanao Island (Hastings *et al*, 2000; Rathod *et al*, 1997). For example, distribution of mefloquine may be a therapeutic option because mefloquine has not yet been used in the Mindanao region, and the results of this study indicated that all Mindanao isolates tested so far were susceptible to mefloquine. Combination therapy with 4-aminoquinolines (chloroquine or amodiaquine) and sulfadoxine-pyrimethamine (Bustos *et al*, 1999; Staedke *et al*, 2001; McIntosh *et al*, 1998) may also be an alternative strategy because sulfadoxine-pyrimethamine is already currently available as the second drug of choice for uncomplicated malaria in the Mindanao region.

This is the first report showing the presence of chloroquine-resistant *P. falciparum* on Mindanao Island by *in vitro* test. To prevent the spread of chloroquine-resistant malaria parasites, current control measures, such as community-based control programs should remain in effect, and control measures based on new prophylaxis or treatment schemes should be carried out simultaneously. In addition, diligent applications of *in vitro* drug susceptibility testing is advantageous in monitoring the distribution of drug-resistant *P. falciparum* in the region.

ACKNOWLEDGEMENTS

The authors wish to thank Dr Tabada, Provincial Health Officer; Dr Romulo Busuego,

Chief of Davao Regional Hospital; and the doctors in the Department of Internal Medicine, Davao Regional Hospital, for their assistance and support. This study was supported by an International Health Cooperation Research grant (11A-4, 13C-5) from the Ministry of Health, Labor and Welfare of Japan.

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