Tropical Medicine and Health Vol. 32 No. 4, 2004, pp. 335-337



# A PILOT FIELD TRIAL OF AN *IN VITRO* DRUG SUSCEPTIBILITY TEST USING THE ANAEROPACK® MALARIA CULTURE SYSTEM ON THE THAI-MYANMAR BORDER

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Accepted 10, September, 2004

**Abstract:** The AnaeroPack® malaria culture system with a portable thermostat incubator was evaluated in a field laboratory on the Thai-Myanmar border conducting *in vitro* drug susceptibility tests on blood samples from 5 Karen children infected with *P. falciparum.* Only one isolate was susceptible to chloroquine; the others were highly resistant. The $IC_{50}$ value of an isolate was only resistant to mefloquine, whereas the values of the 3 patients who presumably showed recrudescence were slightly elevated in the susceptible ranges. These results suggested that chloroquine should no longer be used for *P. falciparum* malaria in this geographic area, and that mefloquine should be carefully monitored for its *in vivo* effectiveness. In this study, the AnaeroPack® malaria culture system with portable thermostatic incubator is a powerful and useful mobile tool, which aids in providing detailed evidence-based distribution data concerning of drug resistant malaria in the field.
**Key words:** AnaeroPack®, Drug susceptibility test, *Plasmodium falciparum*

## INTRODUCTION

Since chloroquine-resistant *Plasmodium falciparum* was first reported in 1959 in Thailand, it has developed resistance to all commonly used drugs [1]. The spread of multi-drug resistant *P. falciparum* is now a major public health problem worldwide, with prophylactic and therapeutic implications [2]. Evidence-based detection of drug-resistant parasites is important for the accurate evaluation of susceptibility to antimalarial drugs. However, isolation of fresh parasites for *in vitro* drug susceptibility testing is often difficult in the field because of the precise experimental conditions needed for the test. *In vivo* susceptibility tests are frequently conducted, but they sometimes fail because of difficulties in following up the patients. In fact, it is sometimes difficult to determine whether an adequate drug concentration has been successfully achieved in the patients' blood. Therefore, *in vitro* tests are indispensable to determine the exact degree of resistance acquired by the parasites.

We previously reported that the AnaeroPack® gas system can be used for the continuous cultivation of both laboratory strains and fresh isolates of *P. falciparum* from patients [3]. This gas system is safer, simpler, and easier to use than the candle jar method. In this study, we evaluated the AnaeroPack® malaria culture system with a portable thermostat incubator in a field laboratory on the Thai-Myanmar border to conduct *in vitro* drug susceptibility tests on *P. falciparum.* The feasibility of the system and the results of the tests are described in this report.

The study was conducted at Rajanagarindra Tropical Disease International Center, a field laboratory center of the Faculty of Tropical Medicine, Mahidol University, located in Suan Phung, Rachaburi, about 200 km west of Bangkok. The area was inhabited by Karen as well as Thai people, and in 2001 more than 6,000 people were subjected to microscopic diagnosis of malaria in the center with about 1,000 positive cases found. Malaria is endemic throughout the year in this area with the peak season occurring around May and June. Mefloquine is currently the first drug of

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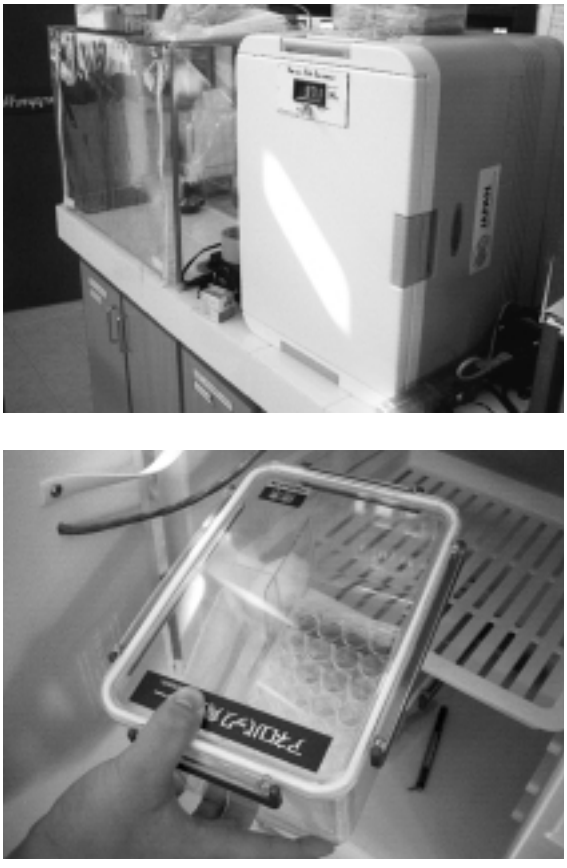
choice for the treatment of uncomplicated malaria, and artesunate, a qinghaosu derivative, is administered in complicated cases.

The AnaeroPack® $CO_2$ (Mitsubishi Gas Co., Tokyo, Japan) is a foil-packed paper sachet that on exposure to air immediately absorbs atmospheric $O_2$ and simultaneously generates $CO_2$ until a condition of 15% $O_2$ and 5% $CO_2$ is attained. The microaerophilic atmosphere produced within a sealed jar (AnaeroPack® Kakugata jar, SUGIYAMA-GEN Co., Ltd., Tokyo, Japan) can be maintained for at least 24 hours. A portable thermostat incubator (SUGIYAMA-GEN Co., Ltd.) was carried to the laboratory, and the temperature inside the incubator was adjusted to 37℃. During *P. falciparum* cultivation, the sachet inside the jar was replaced with a new sachet every day when the culture medium was changed (Fig. 1).

The *in vitro* drug susceptibility test used in this study was a modified semi-micro test described previously [4, 5]. The following procedures were conducted right after the sampling in the field laboratory center. Briefly, blood samples were washed 3 times with RPMI 1640 and resuspended in RPMI 1640 (GIBCO BRL), pH 7.4, supplemented with 10% human serum (from non-immune Japanese donors without a previous history of malaria; Blood Type A) and 25 μg/ml gentamicin (Sigma, Saint Louis, Mo), 25 mM HEPES, and sodium bicarbonate at a hematocrit of 5%. Five hundred microliters of the erythrocyte suspension were placed in each well of a tissue culture plate (24-well flat bottom, Corning Costar, NY). Chloroquine diphosphate was purchased from Sigma. Mefloquine hydrochloride was kindly provided by Hoffman-La Roche Ltd., Basel. Twenty microliters of chloroquine diphosphate or mefloquine was added to each well (for chloroquine to create a series of doubling dilutions from 20 to 10240 nM, and for mefloquine to create a series of 10 times dilutions from 0.01 to 1000 nM dissolved in distilled water). To monitor parasite growth, 6 wells per plate served as controls without antimalarials. When the schizonts were fully grown in the control wells, the culture plate was removed from the incubator. Thin-smear specimens stained with Giemsa solution were made from each well, and we counted the number of erythrocytes microscopically in the control smears until encountering 50 schizonts. We defined the schizonts as parasites that have both dark brown pigment and more than 3 nuclei [6]. The effect of antimalarials on parasite growth was evaluated by observing the decreased number of schizonts per equal number of erythrocytes counted previously in the control cultures. The percentage of growth inhibition effect was calculated as follows: test well schizont count/control well schizont count (50) x 100. Fifty percent growth inhibitory concentration ($IC_{50}$) was calculated from the inhibition curve obtained from the values by a statistical probit method.



(A) (B)

Figure 1. AnaeroPack® culture system. (A) the portable incubator and (B) the AnaeroPack® Kakugata jar with a culture plate inside.

Blood samples were obtained from 5 Karen children age 5 to 13 years (Table 1). The guardians gave written consent to this study, which had been approved by the Ethical Committee of Mahidol University. This survey research also followed the ethical guidelines for epidemiological studies by the Ministry of Education, Culture, Science and Technology and Ministry of Health, Labour and Welfare of Japan. Three of the 5 patients had a history of *P. falciparum* malaria within 1 month, and they had received mefloquine at that time. The parasitemias of the patients were relatively low, 0.05-0.20%. The incubation period (the time needed for the tested isolates to grow to schizonts) ranged from 48 to 96 hrs. The $IC_{50}$ values of the isolates for chloroquine varied from 91 to 402 nM, with a geometric mean of 273 nM and SD of 90 nM. Only one isolate, from patient B, was susceptible to chloroquine; the others were highly resistant. The $IC_{50}$ values of the isolates for mefloquine varied from 4-52 nM, with a geometric mean of 24 nM and SD of 13 nM. The $IC_{50}$ value of the isolate from patient A was only resistant to mefloquine, whereas the values of the 3 patients who

Table 1 Profiles of the *P. falciparum* isolates from 5 patients in Suan Phung

| No. | Age | Sex | % Parasitemia | Day of recrudescence | Chloroquine | | Mefloquine | |
|---|---|---|---|---|---|---|---|---|
| | | | | | $IC_{50}$ (nM) | Judgment* | $IC_{50}$ (nM) | Judgment** |
| A | 12 | female | 0.30 | none | 231 | Resistant | 52 | Resistant |
| B | 13 | male | 0.05 | D29 | 91 | Susceptible | 18 | Susceptible |
| C | 8 | male | 0.20 | D24 | 358 | Resistant | 27 | Susceptible |
| D | 12 | male | 0.11 | none | 285 | Resistant | 4 | Susceptible |
| E | 5 | female | 0.11 | D22 | 402 | Resistant | 17 | Susceptible |

* Threshold of $IC_{50}$ for chloroquine resistance is 114 nM [5].
** Threshold of $IC_{50}$ for mefloquine resistance is 40 nM [9].

presumably showed recrudescence were slightly elevated in the susceptible ranges. These results suggested that chloroquine should no longer be used to treat *P. falciparum* malaria in this geographic area, and that mefloquine should be carefully monitored for its *in vivo* effectiveness.

The World Health Organization's test with the candle jar is the standard method used to cultivate parasites for drug susceptibility testing in the field [7]. This method is sometimes difficult to carry out under field conditions. Our study found the AnaeroPack® system to be an effective alternative method for cultivation of malaria parasites and for *in vitro* drug susceptibility testing in the field. The AnaeroPack® gas system does not need a catalyst, water, or hydrogen for the activation of gas production, and the gas jars or incubator chambers are lightweight and portable. Our findings were consistent with other reports that the AnaeroPack® culture system produced acceptable results for the growth of malaria parasites in the laboratory application. The $IC_{50}$ values attained with the AnaeroPack® $CO_2$ were similar to those attained by the candle jar method [3, 4, 8]. In addition, the thermostatic incubator of AnaeroPack® malaria culture system can now be powered by a car battery. The AnaeroPack® malaria culture system with portable thermostatic incubator is a powerful and useful mobile tool that helps to provide detailed evidence-based distribution data concerning drug resistant malaria in the field.

## ACKNOWLEDGEMENTS

The authors thank the laboratory staff in the Suan Phung field station for their kind assistance and support. This study was supported by a Grant-in-Aid for Scientific Research (B) (International Research) (13576012, 16406012) from the Japan Society for the Promotion of Science, by Asian Centre of International Parasite Control (ACIPAC) project (Hashimoto Initiative) under the Japan International Cooperation Agency (JICA) program, and by a grant from Mahidol University.

## REFERENCES

1. Looareesuwan S, Chongsuphajaisiddhi T. Malaria. In: Looareesuwan S. Wilairatana P, editors. Clinical Tropical Medicine, Bangkok: Medical Media, 1994: 1-61.
2. Alrajhi AA, Rahim I, Akood M, Hazmi M. Chloroquine-resistant *Plasmodium falciparum* cerebral malaria in a chloroquine-susceptible area. J Infect Dis 1999; 180: 1738-41.
3. Mizuno Y, Hatabu T, Kawazu SI, Masuda G, Otomo H, Kano S. Cultivation of *Plasmodium falciparum* isolates under the AnaeroPack gas condition in a portable thermostatic incubator. Jpn J Trop Med Hyg 2000; 28: 383-5.
4. Lin Q, Onda T, Kano S, Masuda G, Suzuki M. *In vitro* drug susceptibility test of *Plasmodium falciparum* using a portable thermostat and $CO_2$ gas. J Jpn Assoc Inf Dis 1999; 73: 1099-103. (Japanese text)
5. Inaba H, Ohmae H, Kano S, Faarado L, Boaz L, Leafasia J, Suzuki M. Variation of incubation time in an in vitro drug susceptibility test of *Plasmodium falciparum* isolates studied in the Solomon Islands. Parasitol Int 2001; 50: 9-13.
6. Silamut K, Phu NH, Whitty C, Turner GDH, Louwrier K, Mai NTH, Simpson JA, Hien TT, White NJ. A quantitative analysis of the microvascular sequestration of malaria parasites in the human brain. Am J Pathol 1999; 155: 395-410.
7. Rieckmann KH, Sax LJ, Campbell GH, Mrema JE. Drug sensitivity of *Plasmodium falciparum* - An *in vitro* microtechnique. Lancet 1978; 7: 22-3.
8. Haruki K, Kobayashi F, Fujino T, Matsui T, Tsuji M. Evaluation of AnaeroPack (AnP) type as tools of *Plasmodium falciparum* cultivation and drug sensitivity tests. Jpn J Trop Med Hyg 2001; 29: 365-70.
9. Milijaona R, Jambou R, Raharimalala L, Ranaivo L, Rason MA, Roux J. Mefloquine-resistant strains of *Plasmodium falciparum* in Madagascar: impact on tourists and public health. Ann Trop Med Parasitol 2000; 94: 313-7.