

## In Vitro Studies of Chloroquine-Resistant Malaria in Thailand

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**OBJECTIVE:** Recent studies in the laboratory and in the field suggest that, within certain limitations, the in vitro method described by Rieckman et al,<sup>1,2</sup> can be used to differentiate between a chloroquine-sensitive and a chloroquine-resistant strain of Plasmodium falciparum. The test is still being modified and requires further evaluation to assess its full value. Therefore, it has been recommended that studies using this test should be conducted in conjunction with the standard in vivo procedure for determining the sensitivity of malaria parasites to chloroquine.<sup>2</sup> Dual testing is not always practical; however, and in three special circumstances, the in vitro test was used alone. The purpose was twofold: to obtain an estimate of the frequency of chloroquine resistant falciparum malaria at three different areas of Thailand and to assess the suitability and certain modifications of the in vitro test for use in field studies.

**DESCRIPTION:** The studies were conducted in Phrabuddhabat, Trad and Nong Khai (see Fig. 1). A total of 194 subjects, all with single infections of P. falciparum, were selected. Most were reporting for treatment at one of the three provincial hospitals or the corresponding Malaria Eradication Center. None of the subjects admitted to having been treated during the 72 hours before they were selected. Many, however, had received earlier treatment, the nature of which could be ascertained in only a few cases, from other medical or paramedical sources. There were 153 male and 37 female subjects (four not recorded) with age ranges of 7 to 62 years (mean 25.3) and 8 to 66 years (mean 25.7) respectively.

Each patient was examined and a blood smear was made to establish a diagnosis, identify the parasite, determine the degree of parasitemia and evaluate the average parasite maturity. The slides were stained with Giemsa and estimates of parasite counts were made by the method of Earle and Perez.<sup>3</sup> Intravenous blood was drawn and the in vitro test was performed as described by Rieckmann et al. Briefly, The test consisted of placing aliquots of parasitized, defibrinated blood in small vials precharged with glucose and zero or graded concentrations of chloroquine. After incubation, thick films were made from the contents of each vial and the parasites were examined. The presence of schizonts with three or more nuclei indicated trophozoite maturation.

Two modifications of the original technique were used. Defibrinated blood with parasitemias in excess of 12,000 parasites per cmm was usually diluted with normal saline to approximately 8,000 parasites per cmm. Secondly, although most tests were set up immediately after defibrination of the blood, eight specimens of defibrinated blood obtained from Nong Khai subjects were kept on wet ice for six hours and transported 40 kilometers over a rough road before aliquots were removed and placed in the culture vials.

The success or failure of each test was dependent on the maturation that occurred in the control vials containing only glucose. When greater than 19 percent of trophozoites matured to schizonts with three or more nuclei the test was considered successful. If the number was less than five percent, the test was unsuccessful. Between 5 and 20 percent, the test was rated as indeterminate because it was impossible to statistically analyze the results. In the successful tests, parasites were considered to be resistant to chloroquine if maturation occurred in any vials containing 0.4 millimicromoles of chloroquine base per ml. or more.

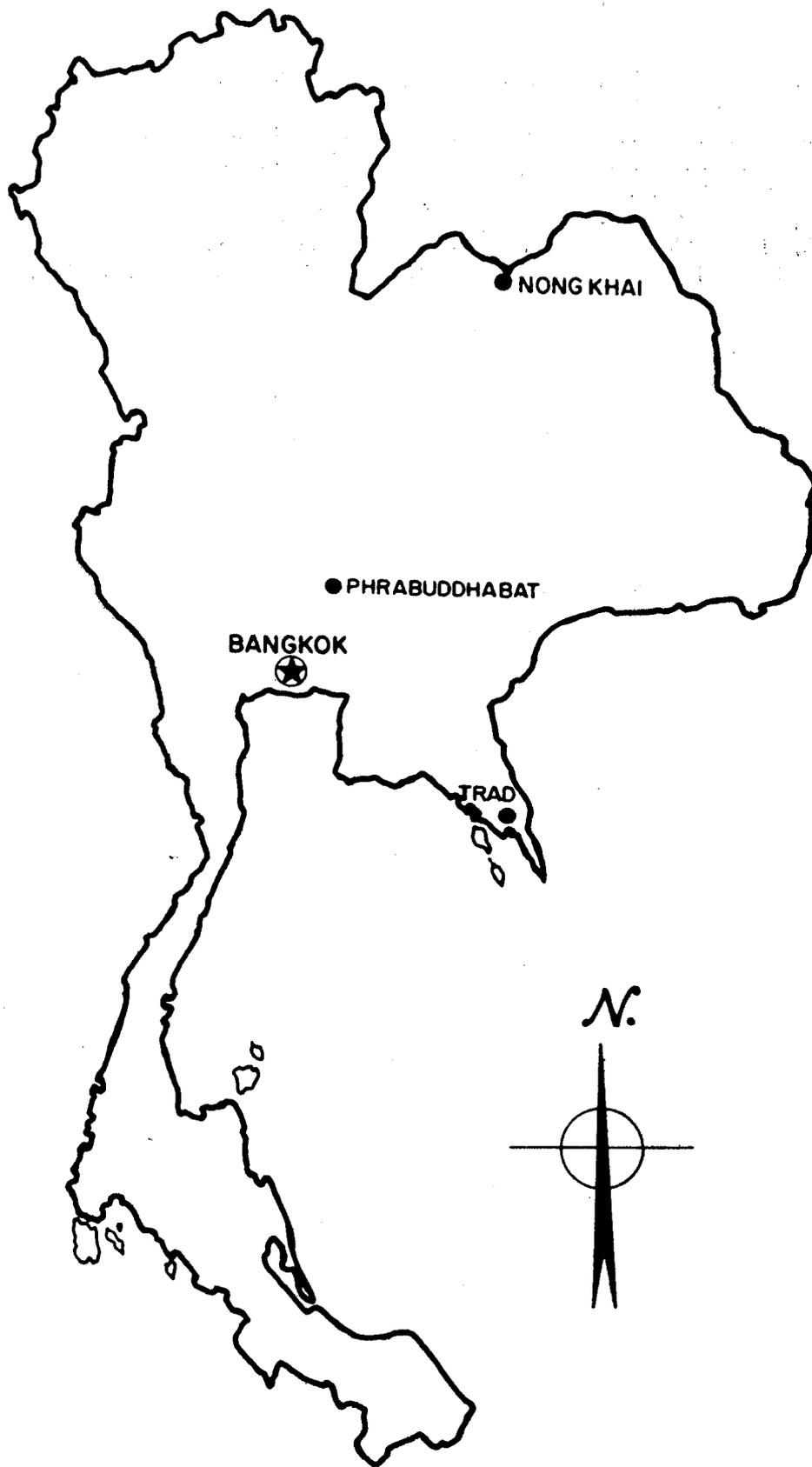
**PROGRESS:** The results obtained with the in vitro test for chloroquine sensitivity are presented in Tables I, II and III. Of a total of 194 tests performed, 38% were successful, 36% were unsuccessful and 27% were rated indeterminate (Table I). Only two of the subjects successfully tested had infections of P. falciparum which were sensitive to chloroquine (Table II). The remainder (97%) were resistant. In the indeterminate group, all but two tests (96%) suggested that the parasites were resistant to chloroquine. These results are similar to those obtained in three recent surveys conducted in vivo in Thailand in accordance with W.H.O. recommendations for a 28 day study.<sup>4</sup> Frequencies of 95<sup>5</sup> and 100<sup>6</sup> percent were reported in Bangkok and 96<sup>7</sup> percent in Phrabuddhabaht. The data suggests that chloroquine-resistant strains of P. falciparum predominate throughout most of central Thailand.

Of the eight tests conducted with blood that had been held for six hours on wet ice and transported to the laboratory, four were successful and indicated chloroquine resistance, three were unsuccessful and one was indeterminate. These results, comparable to the overall success rate shown in Table I, suggest that this technique can be successfully used in field studies. Thus, it may be practical to conduct all in vitro tests in a central laboratory while subjects for study are selected locally and from a wider area by mobile teams operating with a minimum of equipment.

In 67 tests for which normal saline was used to dilute heavily parasitized blood, the percentage of unsuccessful tests was much higher than in the 127 tests performed with undiluted blood (Table IV). Although disappointing, this outcome was not surprising. The use of more physiological diluents is recommended and will be included in any further investigations using the in vitro tests.

The high percentage of unsuccessful and indeterminate tests is unrelated to age or sex of the subject and parasitemia when, in the latter case, the counts do not exceed 20,000 parasites per cmm and the blood is not diluted. Technical problems including bacterial contamination and electrical power failure were responsible for eight unsuccessful tests. It is believed that the principal cause of unsuccessful in vitro tests, other than high parasite densities and technical problems, is the failure to comply with the established requirement for an adequate stage of trophozoite maturity before initiating the cultures.<sup>2</sup> Because this is a subjective determination, further efforts should be directed at more firmly establishing the criteria for identifying trophozoite maturity or, even better, eliminating the requirement altogether.

**SUMMARY:** The frequencies of chloroquine-resistant falciparum malaria as determined by the in vitro test in three locations in Thailand were 95% in Trad, 100% in Phrabuddhabaht and 92% in Nongkhai. It was demonstrated that the in vitro test could be successfully performed with parasitized blood, drawn and defibrinated at isolated locations and transported on wet ice to a central laboratory within six hours. Dilution with normal saline failed to satisfactorily increase the percentage of successful in vitro tests obtained when using heavily parasitized blood. This problem and inadvertent failure to comply with the requirement for trophozoites of adequate maturity remain the two principal causes of unsuccessful in vitro tests which comprise approximately one-third of the tests conducted.



**REFERENCES :**

1. Rieckmann, K.H., McNamara, J.V., Frischer, H., Stockert, T.A., Carson, P.E. and Powell, R.D., 1968. *Am. J. Trop. Med. Hyg.* 17:661-671.
2. Rieckmann, K.H., and Lopez Antunano, F.J., 1971. *Bull. World Health Organ.* (In press).
3. Earle, W.C. and Perez, M. 1932. *J. Lab. Clin. Med.* 17: 1124-1130.
4. World Health Organ. Techn. Rep. Ser., 1968. WHO Expert Committee on Malaria No. 382, pp. 46-48.
5. Harinasuta, T., Suntharasamai, P., Viravan, C., 1965. *Lancet* 2:657-660.
6. Harinasuta, T., Viravan, C., and Reid, H.A. 1967. *Lancet* 1:117-119.
7. Colwell, E.J., Phintuyothin, P., Sadudee, N., Benjapong, W. and Neoypatimanondh, S., 1971. (In manuscript).

**Table I.**  
**Distribution and Classification of the in vitro Chloroquine Sensitivity Test Results.**

Location	Total Tests	Number Successful (%)	Number Indeterminate (%)	Number Unsuccessful (%)
Trad	70	19 (27.1)	21 (30.0)	30 (42.9)
Phrabuddhabaht	91	42 (46.2)	25 (27.5)	24 (26.3)
Nongkhai	33	12 (36.4)	6 (18.2)	15 (45.4)
Total	194	73 (37.6)	52 (26.8)	69 (35.6)

**Table II.**  
**Distribution and Frequency of Chloroquine Resistant Falciparum Malaria as Determined by the in vitro Technique.**

Location	Successful Tests		Indeterminate Tests	
	Number Tested	Number Chloroquine Resistant (%)	Number Tested	Number "Chloroquine Resistant" (%)
Trad	19	18 (94.7)	21	20 (95.2)
Phrabuddhabaht	42	42 (100.0)	25	25 (100.0)
Nongkhai	12	11 (81.7)	6	5 (83.3)
Total	73	71 (97.3)	52	50 (96.2)

Table III.  
Range of in vitro Schizogony Detected in Chloroquine-Resistant  
P. falciparum After Incubation with and without Chloroquine.

Location	Successful Tests		Indeterminate Tests	
	Control Vial (Median)	Chloroquine Vial* (Median)	Control Vial (Median)	Chloroquine Vial (Median)
	Range	Range	Range	Range
Trad	20-93 (36)	5-88 (24)	5-19 (10)	2-27 (7)
Phrabuddhabaht	20-80 (41)	8-79 (29)	5-18 (12)	3-19 (8)
Nongkhal	20-72 (26)	15-16 (24)	6-19 (13)	3-25 (15)

\* 0.4 milimicromoles chloroquine base per ml.

Table IV.  
Effect of Saline Dilution on the Results of the in vitro Tests.

Parasitized blood	Total Number	Number of Successful Tests (%)	Number of Indeterminate Tests (%)	Number of Unsuccessful Tests (%)
Undiluted	127	55 (43.3)	32 (25.1)	40 (31.5)
Diluted with Saline	67	18 (29.8)	20 (29.8)	29 (43.3)