Bulletin of the World Health Organization, 58 (6): 909-912 (1980)

Pyrimethamine sensitivity in *Plasmodium falciparum*: determination *in vitro* by a modified 48-hour test*

P. NGUYEN-DINH [1] & D. PAYNE [2]

Four strains of Plasmodium falciparum *recently isolated in culture were assessed* in vitro *for their response to pyrimethamine. A simple modified 48-hour test was used, which showed two strains to be sensitive to the drug* in vitro, *while the other two were resistant at a very high level. In the two strains for which relevant clinical information was available the* in vivo *response to pyrimethamine was corroborated by the* in vitro *findings. This modified 48-hour test is thus useful for determining patterns of drug sensitivity in laboratory-adapted strains, and would be a valuable asset if found to be equally applicable under field conditions.*

Strains of malaria parasites resistant to pyrimethamine have been reported in many parts of the world (*1*). Their occurrence restricts the use of this valuable antimalarial drug and has now assumed a new importance since pyrimethamine is increasingly used in drug combinations directed against chloroquine-resistant *Plasmodium falciparum* (*2*).

It is now possible, by continuous cultivation techniques (*3*), to maintain different strains of *P. falciparum* in the laboratory, and to test *in vitro* their response to various drugs (*4*), including pyrimethamine (*5*, *6*). A modified 48-hour test that is much simpler than the previous methods was described recently (*7*) for the determination *in vitro* of chloroquine sensitivity in laboratory-adapted strains of *P. falciparum*. This modified 48-hour test has now been successfully used to assess the pyrimethamine sensitivity of four newly isolated strains of *P. falciparum*.

MATERIALS AND METHODS

Origins of the strains

Three strains of *P. falciparum* were isolated in January–March 1980 from malaria cases imported into the United States of America. Blood samples from the patients were collected in heparinized tubes and sent to the Center for Disease Control (Atlanta, Georgia) where they arrived within 10 hours. The samples were used to establish the parasites in culture by the standard Petri dish/candle jar method (*8*, *9*). A fourth strain was isolated and started in culture during field studies in Choluteca, Honduras, in January 1980, before being hand-carried to Atlanta, where it was returned to culture after a total transportation period of 36 hours.

In two of the strains the clinical history of the patient provided information on the *in vivo* response of the parasites to pyrimethamine. Strain Africa-1/CDC (clinically pyrimethamine-resistant) was isolated from a 57-year old white female who acquired *P. falciparum* malaria during a trans-African safari while under prophylaxis with 25 mg of pyrimethamine weekly. Strain Sierra Leone-1/CDC (clinically pyrimethamine-sensitive) was isolated from a 19-year-old Sierra Leonean female in whom *P. falciparum* malaria was diagnosed 3 weeks after her arrival in the USA; the blood sample was collected before the patient was successfully treated with 50 mg of pyrimethamine daily for 2 days.

The two remaining strains were Kenya-2/CDC, from a case of *P. falciparum* malaria imported from Kenya, and Honduras-1/CDC, from a patient seen during an outbreak of urban malaria in Choluteca, Honduras. No information was available on the *in vivo* pyrimethamine sensitivity of the parasites, since other antimalarials were used to treat the patients.

Test method

The modified 48-hour test described earlier (*7*) was adapted for use with pyrimethamine as follows.

The starting material was prepared by washing uninfected O^+ erythrocytes, resuspending them to

* Requests for reprints should be addressed to Mr D. Payne.

[1] Visiting Associate, Bureau of Tropical Diseases, Center for Disease Control, Public Health Service, Department of Health and Human Services, Atlanta, GA 30333, USA.

[2] Technical Officer, Research and Technical Intelligence, Malaria Action Programme, World Health Organization, 1211 Geneva 27, Switzerland.

50% in normal medium (RPMI 1640 medium[a] supplemented with HEPES buffer (30 mmol/litre) and 100 ml of O^+ serum per litre), and seeding them with parasites derived from continuous culture lines. The starting parasitaemias thus obtained varied between 10 and 70 parasites per 10^4 erythrocytes.

Aliquots of 20 µl of this starting material were distributed into flat-bottomed 16-mm wells containing 0.5 ml of either normal medium or medium with various concentrations of pyrimethamine (0.3, 0.1, 0.03, 0.01, and 0.003 µmol/litre, prepared from a 100 µmol/litre aqueous stock solution of the drug). The final erythrocyte suspension was thus 2%.

After shaking to ensure resuspension and uniform resettling of the erythrocytes, the trays were placed in a candle jar and allowed to incubate for 48 hours at 37 °C. After 24 hours the cells were resuspended by agitation of the trays and the gas phase in the candle jar was regenerated.

Table 1. Response (parasite count per 10^4 erythrocytes) of strain Sierra Leone-1/CDC to pyrimethamine in the modified 48-hour test

Concentration of pyrimethamine[a] (µmol/litre)	Gametocytes	Rings	Trophozoites	S = 2N[b]	S>2N[c]	Total count
Time 0	0	9	2	1	3	15
At 48 h:						
Control	0	78	19	6	6	109
	1	79	18	8	16	122
0.3	1	0	5	0	0	6
	0	0	1	0	0	1
0.1	3	0	4	1	0	8
	1	0	8	0	0	9
0.03	1	3	7	3	5	19
	0	4	3	0	4	11
0.01	0	37	10	6	10	63
	1	29	16	12	14	72
0.003	1	60	19	2	22	104
	0	90	4	5	13	112

[a] Duplicate wells were used for each concentration of pyrimethamine.

[b] Schizonts with two nuclei.

[c] Schizonts with three or more nuclei.

[a] GIBCO Laboratories, Grand Island, New York, USA. (Use of trade names or commercial sources does not constitute endorsement by the Public Health Service of the US Department of Health and Human Services.)

Parasite counts were made on Giemsa-stained thin smears taken at the beginning and end of the experiment. Both normal and abnormal parasites were included in the count.

RESULTS

The 48-hour test distinguished two patterns of response to pyrimethamine.

1. Strains Sierra Leone-1 and Kenya-2 were found to be pyrimethamine-sensitive in a series of experiments which yielded consistently similar results. In both Sierra Leone-1 (5 experiments) and Kenya-2 (3 experiments), complete inhibition of the parasite growth was observed in medium containing 0.03 µmol/litre of pyrimethamine. At this drug concentration no increase in parasitaemia occurred over 48 hours, and most of the parasites present were abnormal late trophozoites and schizonts, with only rare rings observed. No clear-cut effect on the gametocytes was apparent. Results from a typical experiment with Sierra Leone-1 are shown in Table 1, and representative curves for both Sierra Leone-1 and Kenya-2 are illustrated in Fig. 1.

2. Strains Africa-1 and Honduras-1 were found to be pyrimethamine-resistant. In both Africa-1 (5 experiments) and Honduras-1 (4 experiments), no inhibition was observed up to the maximal level of 0.3 µmol/litre (Table 2 and Fig. 1). Additional experiments using higher concentrations of the drug showed increases in parasitaemia in medium containing up to 3 µmol/litre of pyrimethamine, with absence of growth occurring only at 10 µmol/litre.

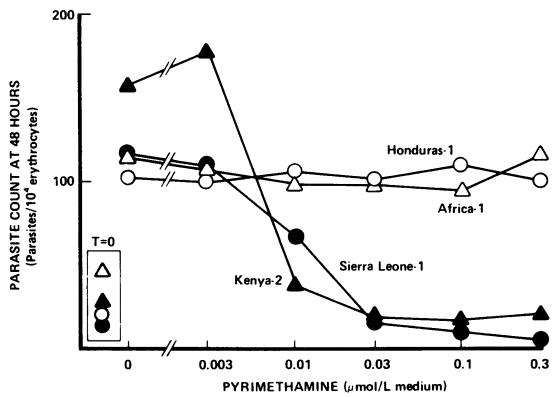

Fig. 1. Pyrimethamine response curves of strains Africa-1/CDC, Honduras-1/CDC, Sierra Leone-1/CDC, and Kenya-2/CDC, in the modified 48-hour test. Each point represents the mean of two duplicate wells. The inset (T = 0) indicates the starting levels of parasitaemia.

Table 2. Response (parasite count per 10^4 erythrocytes) of strain Africa 1/CDC to pyrimethamine in the modified 48-hour test

Concentration of pyrimethamine[a] (μmol/litre)	Gametocytes	Rings	Trophozoites	S = 2N[b]	S>2N[c]	Total count
Time 0	0	27	15	0	4	46
At 48 h:						
Control	2	38	62	9	9	120
	3	47	45	3	10	108
0.3	3	52	56	3	18	132
	6	30	49	7	10	102
0.1	6	29	55	6	5	101
	3	22	53	7	4	89
0.03	7	28	61	12	4	112
	2	20	49	4	8	83
0.01	3	33	48	5	12	101
	1	18	65	5	5	94
0.003	4	39	58	4	10	115
	1	32	53	4	9	99

[a] Duplicate wells were used for each concentration of pyrimethamine.

[b] Schizonts with two nuclei.

[c] Schizonts with three or more nuclei.

DISCUSSION

This work confirms the value of the 48-hour test for assessing *in vitro* the drug sensitivity of laboratory-adapted strains of *P. falciparum*. Two different patterns of response to pyrimethamine were observed. Resistance to pyrimethamine, when present, was marked, the inhibition of the resistant strains occurring only at drug concentrations much higher than those required for the sensitive strains. This confirms observations made previously by Desjardins et al. (*5*) using a different *in vitro* method.

In the two cases where a clinical history made available the relevant information, a good correlation was found between the *in vivo* and *in vitro* responses of the parasites to pyrimethamine.

The 48-hour duration of the test resulted in the parasites being exposed to the drug for a full cycle of asexual multiplication. This made it possible to study the effects of the drug on the morphology of all the parasite stages. Pyrimethamine appeared to act mainly on the late trophozoite and schizont stages, as found by Gutteridge & Trigg (*10*) in earlier *in vitro* studies using *P. knowlesi*. In the present study the decrease in the number of ring stages resulted from the inability of the abnormal schizonts to mature and release viable merozoites, and constituted a valid indicator of inhibition by pyrimethamine. The apparent absence of any effect of the drug on gametocytes was in accordance with the earlier *in vivo* observations by Burgess & Young (*11*).

The possibility of adapting this simple test to field conditions needs to be explored, since a field method for the *in vitro* detection of pyrimethamine resistance is not currently available. The recent spread of chloroquine resistance to Africa (*12*) will result in a greater use of alternative drugs, such as combinations including pyrimethamine. Thus, a field test which could be used with antimalarials of diverse modes of action would be very valuable.

ACKNOWLEDGEMENTS

We thank Dr C. C. Campbell for his participation in the isolation and establishment in culture of the strains studied, and Dr R. E. Desjardins, Wellcome Research Laboratories, for providing the pyrimethamine powder. This work was supported by a grant from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

RÉSUMÉ

SENSIBILITÉ DE *PLASMODIUM FALCIPARUM* À LA PYRIMÉTHAMINE: DÉTERMINATION *IN VITRO* AU MOYEN D'UNE ÉPREUVE MODIFIÉE DE 48 HEURES

Une épreuve modifiée de 48 heures a été employée pour déterminer *in vitro* la sensibilité à la pyriméthamine de 4 souches de *Plasmodium falciparum* récemment isolées et maintenues en culture continue. On a réparti dans des cupules à fond plat de 16 mm des fractions de 0,5 ml soit de milieu normal (RPMI 1640 additionné de 30 mmol/litre de tampon Hepes et de 10% de sérum humain), soit de milieu contenant diverses concentrations de pyriméthamine. Puis,

on y a ajouté des fractions de 20 µl d'une suspension à 50% d'érythrocytes infectés par *P. falciparum* (parasitémie: 0,1 à 0,7%). Après incubation dans une cloche à bougie à 37°C pendant 48 heures, l'accroissement de la parasitémie a été mesuré pour évaluer l'action inhibitrice du médicament. Deux des souches (Sierra Leone-1/CDC et Kenya-2/CDC) se sont montrées sensibles à la pyriméthamine *in vitro*, la parasitémie n'ayant pas augmenté en présence de 0,03 µmol/litre. Les 2 autres souches (Africa-1/CDC et Honduras-1/CDC) étaient hautement résistantes, l'inhibition ne s'étant exercée qu'à une concentration de 10µmol/litre. Pour les souches Sierra Leone-1 et Africa-1, on disposait de données cliniques qui ont permis de constater une bonne corrélation entre les réponses à la pyriméthamine *in vivo* et *in vitro*. La simplicité d'exécution de cette épreuve la place en bonne position pour des essais d'application sur le terrain, d'autant plus que la pyriméthamine fait de plus en plus fréquemment partie des associations de médicaments utilisées contre le paludisme résistant à la chloroquine.

REFERENCES

1. PETERS, W. *Chemotherapy and drug resistance in malaria.* London and New York, Academic Press, 1970, pp. 394-423.
2. WHO Technical Report Series, No. 529, 1973, (*Chemotherapy of malaria and resistance to antimalarials*: report of a WHO scientific group), pp. 24-26.
3. TRAGER, W. & JENSEN, J. B. Human malaria parasites in continuous culture. *Science*, **193**: 673-675 (1976).
4. TRAGER, W. ET AL. Antimalarial activity of *S*-isobutyl adenosine against *Plasmodium falciparum* in culture. *FEBS Letters*, **85**: 264-266 (1978).
5. DESJARDINS, R. E. ET AL. Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrobial agents and chemotherapy*, **16**: 710-718 (1979).
6. RICHARDS, W. H. G. & MAPLES, B. K. Studies on *Plasmodium falciparum* in continuous cultivation. I. The effect of chloroquine and pyrimethamine on parasite growth and viability. *Annals of tropical medicine and parasitology*, **73**: 99-108 (1979).
7. NGUYEN-DINH, P. & TRAGER, W. *Plasmodium falciparum in vitro*: determination of chloroquine sensitivity of three new strains by a modified 48-hour test. *American journal of tropical medicine and hygiene*, **29**: 339-342 (1980) (In press).
8. JENSEN, J. B. & TRAGER, W. *Plasmodium falciparum* in culture: use of outdated erythrocytes and description of the candle jar method. *Journal of parasitology*, **63**: 883-886 (1977).
9. JENSEN, J. B. & TRAGER, W. *Plasmodium falciparum* in culture: establishment of additional strains. *American journal of tropical medicine and hygiene*, **27**: 743-746 (1978).
10. GUTTERIDGE, W. E. & TRIGG, P. I. Action of pyrimethamine and related drugs against *Plasmodium knowlesi in vitro*. *Parasitology*, **62**: 431-444 (1971).
11. BURGESS, R. W. & YOUNG, M. D. The development of pyrimethamine resistance by *Plasmodium falciparum*. *Bulletin of the World Health Organization*, **20**: 37-46 (1959).
12. CAMPBELL, C. C. ET AL. Chloroquine-resistant *Plasmodium falciparum* from East Africa: cultivation and drug sensitivity of the Tanzanian I/CDC strain from an American tourist. *Lancet*, **2**: 1151-1154 (1979).