Development of a blood culture medium and a standard *in vitro* microtest for field-testing the response of *Plasmodium falciparum* to antifolate antimalarials

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The history of the development of the WHO standard in vitro *microtest is summarized, and the rationale and methodology that were used to broaden the system for the testing of antifolate drugs such as sulfadoxine and pyrimethamine (in addition to chloroquine, mefloquine, quinine and amodiaquine) are explained. Additional information is given concerning the composition of the basic and replenishment test kits and the means of procuring them.*

Introduction

The announcement by Rieckmann et al. (*1*) in 1978 of an *in vitro* microtest for the determination of *Plasmodium falciparum* sensitivity to antimalarial drugs and further work by Wernsdorfer et al. (*2*) led to the large-scale production in 1981, under WHO sponsorship, of a standard *in vitro* microtest kit for assessing the response of *P. falciparum* to antimalarial drugs.[a] Performance of this microtest at field level was largely facilitated by the development by WHO of a simple but highly effective technique whereby small quantities (12 ml) of the RPMI-1640 blood culture medium could be reconstituted, as required, in the field from aluminium foil minipacks of the dry-powdered medium (125 mg) and prepackaged stock solutions of HEPES buffer and sodium bicarbonate.[b] The minipacks were specially manufactured for WHO by Gibco Ltd, Scotland (formula number 78-5397).

Initially, the microtest kit was restricted to chloroquine and mefloquine, but subsequently the test range was expanded to include quinine, quinidine and amodiaquine.[b] Alternative tests for pyrimethamine were developed by Nguyen-Dinh & Payne (*3*) and Yisunsri & Rieckmann (*4*) using standard RPMI-1640. About this time (1981), workers in Thailand presented *in vivo* evidence of increasing numbers of *P. falciparum* infections that were resistant to chloroquine and also failed to respond to treatment with pyrimethamine/sulfadoxine in combination (*5*, *6*). Soon after, similar reports came from East Africa (*7*). There was thus a need for an *in vitro* field test for the two classes of compounds which may be called collectively the antifolate antimalarials, i.e., the para-aminobenzoic acid (pABA) competitors (sulfonamides and sulfones) and the dihydrofolate reductase (DHFR) inhibitors (pyrimethamine, cycloguanil and trimethoprim).

Meanwhile, Desjardins and co-workers (*8*) had drawn attention to the problems presented by the testing of antifolate antimalarials using the standard RPMI-1640 blood culture medium (containing 1.0 mg each of folic acid and pABA per litre, as specified by the Tissue Culture Standards Committee in 1974. As pABA is the natural substrate for dihydropteroate synthetase, it is considered necessary that its presence should be reduced to a level near that found in the blood of the human host, and in experiments conducted by Desjardins' group and others in Thailand and Kenya (*9*, *10*), this indeed proved to be the case. The same group also demonstrated that folic acid in the culture medium interfered with the activity of sulfonamides and pyrimethamine *in vitro*. The extent to which the activity of the drug was antagonized by these factors was found to be isolate-dependent and in susceptible *P. falciparum* the amount of these factors occurring naturally in the plasma/serum of the test patient and

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[a] **Payne, D.** *The practical aspects of the standard WHO* in vitro *macro- and microtest systems for the determination of the sensitivity of* Plasmodium falciparum *to chloroquine, mefloquine, amodiaquine and quinine*. Unpublished WHO document/MAP/84.2, 1984.

[b] **Payne, D.** *Report on the development of the WHO standard microtest kit for chloroquine, mefloquine, amodiaquine and quinine 1979–1985*. Unpublished WHO document/MAP/86.1, 1986.

Table 1: **Evaluation and quality control tests of batches of RPMI-1640 (LPLF) liquid medium against standard RPMI-1640**

Purpose of study	Place of study	Production batch of RPMI-1640 (LPLF)	Test period (hours)	Date of test	Comparative indices of asexual parasite growth (%)[a]	
					RPMI-1640 (standard)	RPMI-1640 (LPLF)
Preliminary evaluation	Zambia (field)	Batch I	48 (reinvasion)	Nov. 83	9.2	10.1
Evaluation	Haiti (field)	Batch I	26 (schizont maturation)	Feb. 84	*59.0*	*60.2*
Evaluation	Switzerland (IRTC)[b]	Batch II	48 (reinvasion)	May 85	13.1	13.8
Evaluation	Thailand (field)	Batch II	25 (schizont maturation)	Jul. 85	*38.2*	*42.7*
Evaluation	England (LSHTM)[b]	Batch II	72 (reinvasion)	Jan. 86	4.9	7.0
Quality control	England (LSHTM)	Batch III	72 (reinvasion)	Feb. 87	15.1	14.2
Quality control	USA (CDC)[b]	Batch III	96 (reinvasion)	May 87	8.9	10.2
Quality control	Switzerland (IRTC)	Batch III	48 (reinvasion)	Dec. 87	5.7	6.3
Quality control	USA (CDC)	Batch IV	96 (reinvasion)	Jan. 88	20.1	18.9
Shelf-life studies (23 months)	England (LSHTM)	Batch II	72 (reinvasion)	Mar. 85	8.9	10.2

[a] All end-points indicate percentage parasitaemia except the four in italics which indicate the percentage schizont maturation.
[b] IRTC: Immunology Research and Training Centre, Switzerland. LSHTM: London School of Hygiene and Tropical Medicine, England. CDC: Centers for Disease Control, USA.

in any supplementary serum/plasma caused a marked reduction in the activity of the antifolate antimalarials, especially sulfonamide, whether alone or in combination with pyrimethamine. Quantitatively, this reduction is subject to large individual variation in view of the wide range of physiological levels of pABA and folic acid in the blood.

Methodology and Discussion

Specification of the modified culture medium

On the basis of the experimental data provided by Desjardins et al. (*8*) and additional unpublished information of pABA levels in human plasma made available at that time by K. Y. Sawada (pABA = 0.1–1.0 μg/l), a dry-powdered modified RPMI-1640 blood culture medium was formulated using mid-range levels of normal plasma concentrations, i.e., folic acid 10.0 μg/l (normal range, 5–20 μg/l) and pABA 0.5 μg/l. These levels outweigh, by 5–20 times, the absolute quantities of pABA and folic acid carried along with the blood into the blood/medium mixture and will therefore nearly eliminate the influence of individual differences between the pABA and folic acid blood levels without neutralizing the response to the test drugs. This modified medium was designated as RPMI-1640 (LPLF) (low pABA/low folic acid) and a trial batch of one litre units of the dry powder was prepared by Gibco Ltd.[c]

Preliminary laboratory studies

One-litre packs of the RPMI-1640 (LPLF) medium in dry powder form were distributed to all workers of the group engaged in the development of a sulfadoxine/pyrimethamine (SDX/PYR) *in vitro* test system. The first results from the field in Zambia, in November 1983, where the collaborative studies were undertaken with the Tropical Disease Research Centre, Ndola, were encouraging and were confirmed in further studies by Dr Nguyen Dinh (Centres for Disease Control, USA) and co-workers from the National Malaria Service (SNEM) in Port-au-Prince, Haiti (*11*) (Table 1).

The reports presented at an informal meeting of workers engaged in the development of the SDX/PYR *in vitro* test system in Bangkok, Thailand, in June 1984 provided more supportive evidence.

[c] Gibco Ltd, Paisley, Scotland.

However, the one-litre dry-powdered packs were evidently not suitable for a field test application and it was decided to modify the presentation and provide smaller units of the complete medium (say, 10 ml aliquots), which would be equally successful as the standard WHO microtest kit.

Presentation of RPMI-1640 (LPLF) for the field test kit

The extremely small amounts of pABA and folic acid in the LPLF powdered medium meant it was physically impossible to ensure homogeneity of the powder when the aliquots were of the size envisaged, i.e., 104 mg for 10 ml of medium; the amounts of pABA and folic acid would then be 5 ng and 100 ng, respectively. The only certain means of ensuring a homogeneous mixture with such small quantities is to employ a liquid medium which could be made up in suitably large volumes (say, 50 litres) and divided as required. Liquid RPMI-1640 has long been available commercially, needing only the addition of sodium bicarbonate (0.2%) and human serum to make the complete medium. However, such presentations (minimum quantity, 100 ml) are given a shelf-life, under continuous refrigerated storage, of only nine months against 36 months for the standard dry powder form.

Such a limitation is acceptable in a field test application where 24 months has been found to be the minimum practical turn-over time. Another logical proposition is to use a lyophilized medium. However, in earlier experiments (present authors, unpublished data), during the development of the WHO standard *in vitro* microtest, reconstituted lyophilized RPMI-1640 medium always failed the prolonged shelf-life studies, three to six months being the usual period of acceptable viability. Successful attempts by Nguyen Dinh and Ren (personal communication) have not, as far as we are aware, been validated by other long-term shelf-life studies.

Since the instability of L-glutamine in solution was responsible for the precocious deterioration of the medium, it was thought logical to attempt to produce small units (ideally 10 ml) of liquid LPLF medium and to extend the shelf-life by using a separate presentation of the L-glutamine complement. The latter would be lyophilized and the complete medium obtained by adding an appropriate volume of the stock RPMI-1640 (LPLF) liquid medium, which contains all the components of the medium, including HEPES and sodium bicarbonate, except for the L-glutamine.

A trial batch (Batch I) of liquid RPMI-1640 (LPLF) was prepared by a reputable firm complete (with the exception of L-glutamine) in 10 ml aliquots in sealed 10 ml serum bottles.[d] Dry L-glutamine was presented separately, 3 mg/vial, equivalent to 300 mg/l medium. The 10 ml aliquot of RPMI-1640 (LPLF) was added to the L-glutamine using sterile procedures to produce 10 ml of complete RPMI-1640 (LPLF). Although initially good results were obtained in the laboratory and the field (see Table 1), the shelf-life of the presentation was found to be unacceptably short and in controlled field trials in Brazil in August 1984 (9 months) it failed to provide any measure of adequate growth.[e]

Previous experience (present authors, unpublished data) with the stock solutions of HEPES and sodium bicarbonate for the microtest had shown that the stability of these weak solutions depended to a large extent on the quality of the glassware containers used (the glass must be alkali-free). It was also inversely related to the volume of the liquid and the area of contact with the internal surface of the container vessel (the more liquid and contact with the glass the lower the stability). At the same time other experiments (Payne & Nguyen-Dinh, unpublished data) showed that RPMI-1640 was degraded by exposure to light. Accordingly it was decided to increase the volume of the stock bottle for RPMI-1640 (LPLF) medium to 100 ml and to use high-specification alkali-free brown glass serum bottles for the stock bottle.

The second production (Batch II) in May 1985 was subsequently proved in laboratory and field tests to be equal to or better than the standard RPMI-1640 in supporting parasite growth (Table 1); later this batch was shown to have a shelf-life of at least two years (Table 1) and thus met the required criterion of viability.

However, field trials in Thailand (Payne, Suebsaeng & Rooney, unpublished data) showed that under extreme field conditions the stock bottle of 100 ml RPMI-1640 (LPLF) was susceptible to contamination by adventitious bacteria when repeatedly drawn upon over a period of time. Accordingly from Batch III onwards, 40 mg gentamycin per litre was introduced during the manufacturing process and, provided that standard sterile procedures were followed, this appeared to solve the contamination problem.

Quality control testing in the laboratory and the field has shown that subsequent batches (III and IV) have the same comparable growth characteristics.

Comparative studies in 1987 by Tan-Ariya et al. (*12*) evaluated four media to determine the optimal

[d] Amimed, Basle, Switzerland.

[e] **Nguyen-Dinh, P. & Payne, D.** *Report on a joint CDC/WHO visit to Bélem, Brazil.* Unpublished CDC/WHO report, 1984.

concentration of pABA and folic acid in the *in vitro* assay of antifolates against *P. falciparum*. These media were:

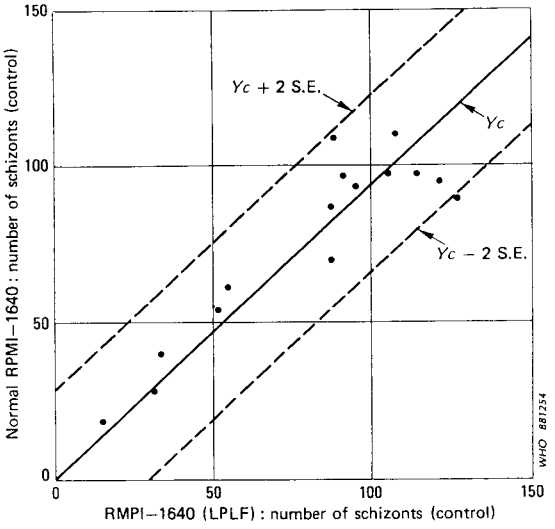
—RPMI-1640 standard formula 430-1800 with 1 mg per litre each of pABA and folic acid;
—RPMI-1640 formula 78-5346 with no pABA or folic acid;
—RPMI-1640 formula AS074-1800 with pABA 0.5 μg and folic acid 10 μg per litre;
—Waymouth medium formula MB752/1 with no pABA and folic acid 40 μg per litre.

They found that the medium containing 0.5 μg pABA and 10 μg folic acid per litre was "the best for parasite growth regardless of the degree of drug sensitivity. Results obtained by using this method agreed most closely with results from *in vivo* observations".

While these confirmatory results were largely obtained with parasite reproduction tests in continuous cultures of *P. falciparum*, earlier work had been carried out to determine how the medium performed in the schizont maturation inhibition test which is the principle of the microtechnique in the field. Such studies were undertaken by several collaborating groups with the result that the RPMI-1640 (LPLF) liquid formulation of Batches III and IV produced on average at least the same schizont maturation in the controls as that achieved with normal RPMI-1640. Paired tests carried out by us with the Malaria Division, Ministry of Health, Thailand (Fig. 1) indicate that the LPLF formulation performs in general slightly better than the normal RPMI-1640. In addition, there were some isolates which showed readable schizont maturation with RPMI-1640 (LPLF) while there was none in normal RPMI-1640. The reverse was not seen. For the isolates which had readable schizont maturation in both media (Fig. 1), the quantitative correlation between the results with RPMI-1640 (LPLF) and normal RPMI-1640 was strong (correlation coefficient $r = 0.91526$; S.E.$_r$ = 0.04501).

The encouraging results with Batch II and confirmatory reports[f,g] by workers engaged in the development of a sulfadoxine/pyrimethamine *in vitro* test system promoted the development of a second version (Mark II) of the WHO standard microtest kit based on the WHO standard RPMI-1640 (LPLF) liquid medium. This kit provides all the necessary material for the field evaluation of five

Fig. 1. **Results of paired schizont maturation tests with fresh isolates of *Plasmodium falciparum* in normal RPMI-1640 and RPMI-1640 (LPLF) culture media (control well readings = number of schizonts counted in the control wells of standard WHO microtest plates. Yc = normal regression calculated from observed values, with ±2 S.E. (standard errors), corresponding to $P \geqslant 0.95$).**



common antimalarial drugs (the four already covered by the first series (chloroquine, mefloquine, quinine and amodiaquine) and sulfadoxine plus pyrimethamine in combination).

The test kits of the second series went into production in October 1987 (see Annex for details) since when the first series has been discontinued. With the exception of the WHO standard RPMI-1640 (LPLF) liquid medium and the WHO standard RPMI-1640 minipack dry medium, all components of the two series of microtest kits are interchangeable.

The introduction of the WHO standard *in vitro* microtest Mark II has permitted rationalization in the standard microtest procedure (eliminating the need for micro-filtration and several of the former manipulatory steps) and provided a 10% reduction in the cost per test.

Résumé

Mise au point d'un milieu de culture et d'un micro-test *in vitro* standard pour la détermination sur le terrain de la réponse de *Plasmodium falciparum* aux antifoliques

Cet article décrit l'historique de la version Mark II du nécessaire OMS standard pour micro-test *in vitro* et indique les modifications techniques ayant

[f] **Subsaeng, L.** *Progress report Feb. 1984 to March 1985*. Centre for Development and Evaluation of Tests for the Assessment of Drug Response of *Plasmodium falciparum*, Malaria Division, Ministry of Public Health, Bangkok, Thailand.

[g] **Rieckmann, K.H.** *Report on a visit to Thailand*. WHO unpublished document, 1984.

conduit à son adoption en octobre 1987 comme nécessaire standard OMS pour micro-test *in vitro*.

Ce nécessaire d'épreuve peut être utilisé pour tester sur le terrain les antipaludiques de la catégorie des antifoliques tels que la sulfadoxine et la pyriméthamine en association ainsi que la gamme de médicaments—chloroquine, méfloquine, quinine et amodiaquine—couverte par l'ancienne version du nécessaire d'épreuve.

L'article décrit les critères techniques qui doivent être remplis et les problèmes qui doivent être surmontés avant de pouvoir mettre au point un milieu de culture convenable pour l'épreuve de sensibilité aux antifoliques; les considérations conduisant au choix d'une période d'incubation de 24 à 30 heures pour la détermination de la maturation des schizontes sont décrites et discutées. L'article donne également des renseignements sur la composition des nécessaires d'épreuve et de leur trousse de réassortiment ainsi que sur les moyens de se les procurer.

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Annex

The WHO standard in vitro *microtest Mark II*

The WHO standard *in vitro* microtest for the assessment of the response of *Plasmodium falciparum* to antimalarial drugs is produced under a non-profit tripartite agreement involving the Philippine Government (National Malaria Service), the WHO Regional Office for the Western Pacific, and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases with the WHO Malaria Action Programme.

The test kits (first series) were produced from 1982 to 1987 and the second series (Mark II) went into production in October 1987. The standardized test material is presented in the form of two test kits.

Kit A (Mark II)

Basic kit containing all the material required to conduct the test under field conditions with the exception of an incubator and a microscope.

The expendable components are sufficient for 144 individual tests (12 test plates).

Cost,[a] inclusive of delivery by air to any place in the world, is US$ 450.

Kit B (Mark II)

Replenishment kit containing the expendable components for 72 individual tests (6 test plates).

Cost,[a] inclusive of delivery, is US$ 150.

The standardized test plates, as supplied with the test kits (and also available separately at US$ 5.00,[a] inclusive of delivery; minimum order, 5 plates) are available for chloroquine, mefloquine, quinine, amodiaquine and sulfadoxine/pyrimethamine. Any

[a] Subject to change owing to inflation/currency exchange rate fluctuations.

combination of these test plates can be supplied with the test kit at the request of the purchaser.

The test kit and test procedure are fully described in the unpublished WHO document MAP/87.2, copies of which are available in English, French and Spanish from WHO.

All enquiries concerning the purchase of the test kits should be addressed to Dr A. Shirai (TDR), WHO Regional Office for the Western Pacific, P.O. Box 2932, Manila 2801, Philippines. (Telex: UNISANTE MANILA 27652. Fax: 632/52 11 036).

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