Field evaluation of drug resistance in malaria: in vitro micro-test

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Field evaluation of drug resistance in malaria.
In vitro micro-test

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Introduction

Experience with mepacrine, dehydrofolate reductase (DHFR) inhibitors and 4-aminoquinolines shows that resistance of malaria parasites to antimalarial drugs has become a widespread, practical problem confronting malarialogists and public health workers in the endemic countries and many a clinician in non-malarious countries which are exposed to the importation of malaria cases from abroad.

Consequently, it is necessary to develop objective methods for assessing the response of malaria parasites to drugs in order to monitor sensitivity, to predict or to detect resistance and, if so indicated, to modify therapeutic approaches.

The standard, 7-day in vivo test (WHO, 1973) has the disadvantage of not differentiating between S and RI responses; the extended, 28-day test implies daily blood examination and a high and rarely obtained measure of patient cooperation. It may also be invalidated by new infections.

The in vitro macro-test for *Plasmodium falciparum* (Rieckmann et al., 1968), now widely used as the WHO Standard Test (WHO, 1979), needs a blood volume of at least 10 ml, to be obtained through venipuncture, and blood samples containing mainly advanced ring stages of an asexual parasitaemia of 1,000–80,000/μl blood. Its applicability in children is seriously limited.

Micro-technique (micro-test)

Employing the elements of the candle jar/petri dish method of continuous in vitro culture (Trager and Jensen, 1976), Rieckmann et al. (1978) have des-
cribed a micro-technique for a 24-hour drug sensitivity test for *Plasmodium falciparum* which they had used with *Aotus* blood. After validating the technique for use with human blood in patients from Thailand in 1978, further evaluation and modification of the system was carried out in Brazil, Colombia and the Sudan (Lopez Antuñano and Wernsdorfer, 1979; Kouznetsov et al., 1979). Present activities aim at the standardization of test procedures and materials with a view to producing test kits which could be used in the global studies on baseline assessment within the framework of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

**Performance**

The test is performed on flat-bottomed tissue culture plates (8 × 12 wells) which are pre-dosed with the appropriate drug. We have so far produced plates with chloroquine and mefloquine – for trial purposes also with pyrimethamine, Dabequin (4-aminobenzo(g)-quinoline), a new drug from the USSR, and Qing hao su, a Chinese drug obtained from *Artemisia annua*. The pre-dosed, sealed plates contain 12 identical series (columns 1–12) of controls and ascending drug doses in dry form.

The in vitro test can be carried out with blood containing at least 500 asexual parasites of *Plasmodium falciparum* per µl. Mixed infections and blood from patients with recent treatment history should be excluded. For the test 100 µl of blood from the finger or earlobe are drawn in a sterile, anticoagulent-treated capillary and ejected in 0.9 ml of freshly prepared (or reconstituted) medium. The blood is suspended by gentle agitation and preferably processed without delay. Storage at 37° C for up to 3 h is permissible.

After removing the seal strips from the appropriate column(s), 50 µl of blood/medium suspension each are added to the wells and the plate is closed with a sterile lid. The plate is gently rocked for solving the drug deposits and then placed in a candle jar or a candle water basin. The vessel is closed after lighting the candle. After incubation at 37.5° C for 24–26 h, the medium is removed from the individual wells and thick films are made from the erythrocyte sediment. After staining with Giemsa, schizont maturation is assessed for controls and drug wells, and is expressed for the drug wells as % of the control values.

**Evaluation**

If the growth in the controls is adequate and the parasites are only *P. falciparum*, the following may be concluded in the case of blood samples containing less than 90,000 parasites per µl:

- total inhibition of growth at 1.14×10⁻⁶ mol chloroquine/1 blood (= 5.7 p-mol/well) indicates susceptibility to standard chloroquine treatment;
- growth at 1.6×10⁻⁶ mol chloroquine or more/1 blood (= 8 p-mol/well) indicates resistance of *P. falciparum* to chloroquine;
preliminary in vitro results with mefloquine in the micro-test indicate that a dose of \(0.8 \times 10^{-6} \text{mol/l blood} (= 4 \text{p-mol/well})\) normally completely inhibits maturation of schizonts.

**Application**

The in vitro micro-test is not meant to replace the in vivo assessment of the response to treatment with chloroquine. However, it provides a useful means for monitoring parasite sensitivity to drugs and for detecting the emergence of drug resistance.

The micro-test has a wider range of application than the in vitro Standard Test (macro-test) since two series of tests, e.g. with chloroquine and mefloquine, can be run with 100 \(\mu\)l blood which can easily be obtained through a finger-prick. The stage of development of the rings is not critical. Damage of the blood samples in transport is very rare since the blood is taken up in medium. There is also apparently no upper limit of parasitaemia at which the test can be applied – blood from patients rarely exceeds 10% parasitaemia. However, reading and evaluation of the test may be impaired at parasite densities exceeding 90,000/\(\mu\)l blood. Growth conditions in the micro-test are better than in the macro-test. This is evident from the morphology of the parasites from the control wells. However, these favourable growth conditions will cause the bursting of schizonts and merozoite invasion of other erythrocytes when the incubation time is prolonged. This invalidates the test which is essentially based on the evaluation of the growth-differential of originally synchronous parasite material.

**Findings**

A comparison between the results of the micro-test and the macro-test was carried out in Prabuddhabat, Thailand, in April 1978, with blood from eight patients. The eight parallel series yielded regression lines of remarkable similarity, with regard to both the base value and the regression coefficient (Fig. 1). It can be concluded that the results of macro-test and micro-test are comparable – larger test series may provide an even closer approximation of the regressions.

Selective uptake of chloroquine by the parasite-infected erythrocyte is particularly evident in the micro-test system which also allows processing of blood samples with high parasitaemia. While we failed to recognize a relationship between the degree of parasitaemia and the inhibition of schizont maturation at parasite densities below 90,000/\(\mu\)l blood, such a relationship was suggested from investigations in Prabuddhabat, Thailand, in chloroquine-resistant isolates, and quite evident from the studies in Sennar, Sudan, where *P. falciparum* is still sensitive to chloroquine (Fig. 2). This phenomenon, probably caused by a quantitative dose/parasite relationship, merits further investigation with isolates of defined sensitivity.

The yield of the micro-test system is generally better than that of the macro-test. It rarely fails when the selection of the patients is properly done. In
Fig. 1. Chloroquine-induced inhibition of schizont maturation in chloroquine resistant \textit{P. falciparum}: comparison of macro-test and micro-test in eight isolates from Prabuddhabat, Thailand. Linear regressions.

Fig. 2. Chloroquine-induced inhibition of schizont maturation in \textit{P. falciparum}: comparison between isolates with $>90,000$ and $<90,000$ asexual parasites per micro-litre, from Prabuddhabat, Thailand (chloroquine resistant), and Sennar, Sudan (chloroquine-sensitive). Linear regressions.
Sennar, Sudan. 29 out of 30 tests were successful (Kouznetsov et al., 1979). The one failure was caused by contamination.

**Further development of the micro-test**

The in vitro micro-test is a most promising tool and preliminary observations indicate that it offers many advantages over the Standard (macro) Test. Before replacing the latter, the micro-test needs to be further developed, the test material standardized, and the test system widely validated.

In the present form, the test can be utilized for 4-aminoquinolines, mefloquine, and probably also for quinine and Qing hao su.

A major obstacle to the production of reliable test plates is the phenomenon of drug adsorption to surfaces of material used during the preparation process such as glass or plastic bottles, vials, pipettes, dosing trays, and tissue culture plates. There are also important differences in the degree of adsorption within specific groups of material, e.g. glass. An example of specific adsorption

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**Fig. 3.** Adsorption of $^{14}$C mefloquine to plastic tissue culture plates as measured from the supernatant on reconstituted dry micro-plates kept at 4°C. Comparison between theoretical regression, observed means, and the ln x ln y power regression derived from $6 \times 25$ observations on days 1, 2, 4, 7, 16 and 32 after dosing (no significant fluctuation between days 1–32, indicating stabilization).
of $^{14}$C labelled mefloquine on plastic, flat-bottomed tissue culture plates, is given in Fig. 3. It appears that an appropriate correction for loss by adsorption is feasible within acceptable limits of variation.

Further studies, conducted within the framework of the Scientific Working Group on the Chemotherapy of Malaria, are being undertaken by several laboratories in order to assess the shelf life of dosed test plates and freeze dried medium under tests, to study inter-plate variation, and to adjust the system to field application.


