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The Evaluation of in vitro Tests for the Assessment of Drug Response in Plasmodium falciparum

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Introduction

Drug resistance of *P. falciparum* has become a problem of concern not only to countries endemic for this species, but also to non-malarious regions where returnees or visitors from the tropics may require urgent diagnosis and treatment. Individual testing for the response pattern of *P. falciparum* has limited scope in view of the need for immediate therapeutic intervention. However, knowledge about the sensitivity situation in the area of acquisition of the infection will play a decisive role in the selection of the appropriate drug and of the regimen to be used. Such knowledge requires the monitoring of the drug response of *P. falciparum,* particulary in malaria-endemic areas.

Available field test systems

The first systematic approach to the assessment of drug response of *P. falciparum* is based on in vivo testing (13), using a standard dose regimen of chloroquine and daily parasitological checking for 7 days (standard test) or for 28 days (extended test). The results permit the classification into sensitive response (S), recrudescence (R-I), overt resistance (R-II), and high resistance (R-III). This type of test can also be run with other antimalarial drugs, provided the period of the post-treatment follow-up is adjusted to the half-life of the medicament employed (11). The in vivo tests suffer from various disadvantages, especially the need for regular checking over a relatively long time period, the strong influence of immunity on its outcome, the purely qualitative nature of its results, and eventual clinical deterioration in the case of R-III responses.

Based upon the phenomenon of sequestration and the observation that glucose-supplemented *P. falciparum* can be held in short-term culture for up to 48 hours (1), RIECKMANN et al. (6) developed an in vitro test system in which natural isolates of *P. falciparum* in defibrinated blood were exposed to various drug concentrations for 24 - 26 hours, time enough for the formation of schizonts. The drug-related impact on parasite growth yielded usually a cut-off point and a measure of the relative inhibition of schizont maturation. Although quite useful, this "macro-test" has the disadvantage of necessitating venepuncture.

Soon after the achievement of the continuous cultivation of *P. falciparum* (9) its elements were employed in the development of a micromethod (7) that has been adapted to field use (10). It requires only a small volume of blood that can be drawn by finger puncture.

Performance of the in vitro micro-test

The tests are usually conducted with the material contained in the WHO standard test kit for the assessment of the in vitro response of *P. falciparum* to chloroquine, amodiaquine, quinine, mefloquine and sulfadoxine/pyrimethamine (14). The test kits contain all essential material in a standardized, quality-controlled form. They are available through the WHO Regional Office for the Western Pacific, Manila, Philippines.

For the performance of the test, 100 µl of blood are taken into a sterile, heparinized capillary tube and added 10 900 µl of the culture medium RPMI-1640 LPLF. After gentle agitation for homogeneous suspension, 50 µ aliquots are distributed into the wells of the scheduled column of a pre-dosed mictotitre plate. These pre-dosed, drug-specific plates contain 12 columns of 8 wells each. The first row of wells (A) is free of drug and serves as a control. Rows B – H contain ascending quantities of the drug.

After closing the microtitre plate with a sterile lid, and gentle agitation for the reconstitution of the drug deposits, the plate is placed into a candle jar (desiccator), the candle is lit, and the jar's cover closed. After extinction of the candle, the jar is placed into an incubator set at 37.5 – 38.0° C, and held there for 24 – 26 hours.

After incubation the tests are "harvested" by removing the medium-plasma supernatant form each well, and placing the sediment as a thick film on a microscope slide. This is done in a set order so that all 8 thick films of one test can be placed on one duly marked slide.

After thorough drying, the slide is stained with Giemsa solution, using a dilution of approximately 1 : 50 to 1 : 70 of commercial stock solution, at pH 6.8 for 30 – 45 minutes.

Reading of the in vitro micro-test

The reading commences with the control (well A), enumerating schizonts (≧ 3 chromatin dots) and other asexual forms separately, to a total of 200. If the count shows a total of ≧ 20 schizonts, or ≧ 10% of all asexual parasites, the test is valid, and the reading can proceed with wells B – H. (NB: in tests with sulfadoxine/pyrimethamine only schizonts with ≧8 normal chromatin dots are counted as "schizonts" as there is little inhibition during the trophozoite phase.)

Evaluation of in vitro micro-tests

The early evaluation of the in vitro tests pertained mainly to the cut-off point of schizont maturation, i. e. the first drug concentration that yielded a complete inhibition of schizont maturation. Later it was realized that the quantitative inhibition pattern shows linearity when the drug concentrations are expressed as logarithms and the concentration-specific inhibitions as probits. Based on this log-normal distribution and the classical work of LITCHFIELD and WILCOXON (5), a method has been described for the electronic processing of drug sensitivity data that yielded the main regression parameters and the key effective concentration (EC) values (3). However, this programme was not geared to provide the parameters required for longitudinal and geographical comparisons of drug sensitivity. In the absence of suitable commercial computer programmes these continued to require cumbersome calculations.

Preliminary evaluation

The individual test will usually show descending numbers of schizonts the higher the drug concentration in the well. Within the common dose range of the plates there will be with most drugs also a "cut-off point" at which schizont maturation has ceased. The situation of the cut-off point gives a rough indication of the isolate's response. Thus, complete inhibition of schizont maturation in well D of the chloroquine plate (0.8 µl/l blood) can be taken as a reliable sign of sensitivity, whereas schizont maturation in well E (1.6 µ/l blood) indicates resistance.

Also grouped data should be examined for the cut-off points. In table 1 two series of sensitivity tests (chloroquine) from the same area in East Africa are summarized. There were 12 sensitive isolates out of 41 in 1988, with 22 borderline responses, while 7 isolates showed

Table 1:

Cut-off points of schizont maturation with chloroquine in *Plasmodium falciparum* from East Africa, 1988 (n = 41) and 1994 (n = 44)

Chloroquine concentration µmol / l blood	Number (%) of isolates showing complete inhibition of schizont maturation	
	1988	1994
0.2	0 (0)	0 (0)
0.4	0 (0)	0 (0)
0.8	12 (29)	0 (0)
1.6	34 (83)	9 (20)
3.2	39 (95)	23 (52)
6.4	41 (100)	31 (70)
12.8	41 (100)	44 (100)

Table 2:

Key parameters of chloroquine sensitivity in 44 isolates of *Plasmodium falciparum* from an area in East Africa, 1994, comparing unweighted series and data standardized for ∑C/N.

Parameter	Unweighted series	Standardized series
Intercept a	4.8017	5.0025
Slope b	0.0987	0.9330
EC_{50} µmol / l	1.2242	0.9973
EC_{90} µmol / l	4.5353	3.9390
EC_{99} µmol / l	13.1927	12.0689
Chi^2 for heterogeneity	1.0393	0.7103

manifest resistance. In 1994 there were 9 borderline cases out of 44 isolates, the remainder showed resistance. Such tables lend themselves also to a simple quantitative evaluation in the form of a 2 × 2 test with a suitable intersection point, or by a multiple table Chi^2 test. Using a multiple table test in the above-cited example Chi^2 came to 35.0198 (or $p < 0.0005$ at N = 3), indicating that there has been a highly significant loss of chloroquine sensitivity between 1988 and 1994.

While being suitable for a summary overview of the sensitivity situation, the analysis of the cut-off points does not provide any parameters of the parasite population's response below the cut-off points which is best characterized by certain key EC values and the slope of the concentration-response regression.

Quantitative Analysis

Practically all *P. falciparum* populations without very recent drug contact show a log-concentration normal distribution with regard to enzyme-inhibiting compounds. Therefore, the evaluation of single or grouped isolates should follow the classical method of LITCHFIELD and WILCOXON (5) for the evaluation of dose-effect studies, adopting a mathematical rather than a nomogrammatic approach. Recent drug pressure which could interfere with the normality is usually associated with poor schizont maturation in the controls, invalidating the tests with such isolates.

The response patterns of individual isolates may be of clinical interest and useful in sentinel situations, but in the wider epidemiological context, collective area- and time-specific data carry far more importance.

Commonly, simple addition of the schizont counts is used for pooling the test data. However, recent insight in the biological advantage or disadvantage of parasites resistant to specific antimalarial drugs (12) suggests that the individual test results should be standardized for the controls (∑C/N), thus avoiding an unduly high weight of isolates showing abundant schizont maturation, and the underrating of isolates with moderate growth. The same is achieved by converting the individual readings into percentages of the control counts and using these as a basis for the further calculations. This is illustrated on the example of the 1994 chloroquine test series mentioned earlier (Table 1). Based on the unweighted series the EC_{50} and EC_{90} values appear to be higher than those obtained after standardization (Table 2). This is obviously the result of a biological advantage of chloroquine-resistant *P. falciparum* (12).

Standardization for equal weight of the individual isolates appears therefore to be a necessary precaution for avoiding distorted overall results. This is particularly important in longitudinal observations where, in the absence of standardization, the relative distortion would increase with the rise of drug resistance. The authors have therefore included such standardization in the new computer programme.

For regression analysis, the log-concentration normal distribution of drug response necessitates the transformation of the concentration data (x) into logarithms and the transformation of the response data (y) into probits (i. e. standard deviations from the mean, or probit

0 = EC_{50}). When processed according to ordinary linear regression analysis (8) this would yield a straight regression line, ususally conforming with the "least squares" fit to the observed data points. However, the ordinary linear regression does not take into account the relative variability of data points which, beyond probit 0 ± 1, rises with increasing distance from the mean. LITCHFIELD and WILCOXON (5) have made appropriate allowance for this phenomenon by allocating proportionally decreasing weight to the data points beyond probit 0 ± 1 (i. e. data outside the EC_{16} - EC_{84} range). Applying this principle to the analysis of drug sensitivy tests in *P. falciparum* yields generally a better curve fit than that obtained with the unadjusted method (see Chi^2 values for heterogeneity in Table 2).

The new computer programme, based on the stipulations of LITCHFIELD and WILCOXON (5) starts with the input of the raw data (Table 3), standardizes the data for equal weight of all isolates, transformes the concentration data to logarithms and the inhibition data to probits. From there it processes the regression and calculates all key parameters (Table 4).

These key parameters are (in accordance with [5]):

n	Number of isolates tested
a	Intercept of regression (based on probit + 5)
b	Slope of regression
r	Correlation coefficient of regression
Chi^2	Chi-square for heterogeneity (acceptable limit depending on K)
S	Slope function
A	Intermediate term for the calculation of f_s
K	Number of drug concentrations tested, incl. first with 100% inhibition
N'	Number of data points between EC_{16} and EC_{84}
R	Highest/lowest drug concentration tested (as per K)
f_s	Factor of S
f_{EC}	Multiplication/Division factor for obtaining 95% confidence intervals of relevant EC
EC_1,	EC_{16}, EC_{50}, EC_{84}, EC_{90}, EC_{95}, EC_{99} with 95% confidence intervals
SMI	% inhibition of schizont maturation at given drug concentration.

It could be argued that, especially with series representing important interindividual variation of drug response, the collective EC_{50} should be established on the basis of the mean of the individual EC_{50} readings. The example in Tables 3 and 4 represents such a situation. Here, the geometric mean of the individual EC_{50} readings comes to 0.9511 µmol / l, with the 95% confidence intervals between 0.7870 and 1.1493 µmol / l as against an EC_{50} of 0.9973 µmol / l and 95% confidence intervals of 0.7713 to 1.2896 µmol / l with the pooled data which provide a more conservative and probably more realistic picture. In practice, it may be impossible to obtain sufficiently reliable calculation of all individual EC_{50} values.

Comparison of Regressions

While the earlier programme (3) yields the basic parameters for intercept (a) and slope (b) as well as a useful range of EC values, it does not provide the parameters for the comparison of regressions. Such comparisons can be of special interest in longitudinal studies (2) or in oeco-epidemiological investigations (4). The new programme furnishes the elements required for the tests for parallelism and activity differences (5).

This may be illustrated by the detail parameters of the study series mentioned in Table 1. These are shown in Table 4 for the 1994 data, and in Table 5 for the 1988 data.

The test for parallelism requires the calculation of Slope Ratio (SR), and factor of the Slope Ratio (f_{SR}). The basic parameters for these calculations (Slope Function S and Factor of the Slope Function f_s) are shown in the print-out.

$SR = S_1 / S_2$ where S_1 is the larger value.

$$f_{SR} = \text{antilog} \sqrt{(\log f_{s1})^2 + (\log f_{s2})^2}$$

If $f_{SR} > SR$ the two regression lines can be considered parallel within experimental error. In the example of the series of Tables 4 and 5 SR = 1.2992 and f_{SR} = 1.3128. Therefore $f_{SR} > SR$, and the two regressions may be compared for an efficacy difference. For this purpose the Potency Ration (PR) and the Factor of the Potency Ration f_{PR} are calculated:

$PR = EC\text{-}50_1 / EC\text{-}50_2$ where $EC\text{-}50_1$ is the larger value.

$$f_{PR} = \text{antilog} \sqrt{(\log f_{EC\text{-}50_1})^2 + (\log f_{EC\text{-}50_1})^2}$$

When $PR > f_{PR}$ the EC_{50} values can be considered significantly different at the 95% confidence level. In the example of the regressions of Tables 4 and 5, PR = 2.2942 and f_{PR} = 1.3856. Therefore $PR > f_{PR}$ indicating a significant loss of chloroquine sensitivity between 1988 and 1994. Comparison is also possible at other EC levels, employing the corresponding f_{EC} figures from the computer output.

The regression lines and their confidence intervals lend themselves to graphic display as shown in Figure 1 (data of the two study series of Tables 4 and 5).

The programme is user-friendly as it only requires the input of the raw data from the schizont readings and provides an output with all key parameters, including those required for subsequent comparisons.

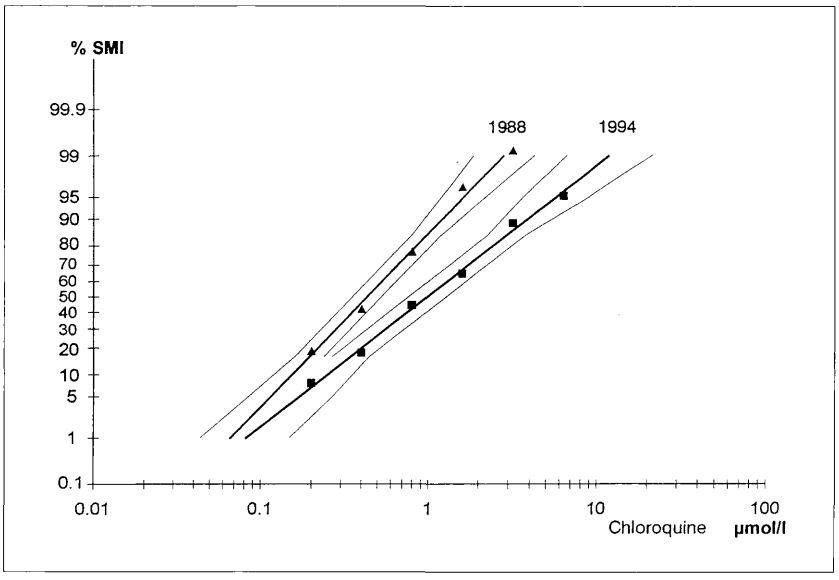

Figure 1:

Log concentration / response probit regressions and their 95% confidence intervals showing the chloroquine sensitivity of *Plasmodium falciparum* in an area of East Africa, 1988 (n = 41) and 1994 (n = 44). % SMI = % inhibition of schizont maturation.

Summary

In vivo tests for the drug sensitivity of *Plasmodium falciparum* do not permit a precise, quantitative assessment of the parasite's response. In contrast, field-adapted in vitro tests measuring drug-related inhibition of schizont maturation are suitable means for the exact determination of drug sensitivity. A standardized micro-method, using quality-controlled material, is currently most widely used for in vitro testing of *P. falciparum*. While an evaluation of the cut-off points of schizont maturation provides a quick general orientation with individual and grouped isolates, it does not replace an exhaustive quantitative analysis. For this purpose the log-concentration/response probit approach is obviously the most appropriate analytic procedure in view of the log-normal distribution of drug response in natural populations of *P. falciparum*. The paper introduces a task-oriented computer adaptation of the method.

Key words

Plasmodium falciparum, drug sensitivity, data analysis.

Table 3:

Computer input of raw data from 44 in vitro sensitivity tests with chloroquine in an area of East Africa (E-group 2), 1994.

A	B	C	D	E	F	G	H
Control schiz	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ
0	0.2	0.4	0.8	1.6	3.2	6.4	12.8
163	197	168	132	96	37	0	0
57	43	42	11	0	0	0	0
136	133	110	94	50	10	0	0
38	26	18	6	0	0	0	0
83	81	79	36	18	2	0	0
177	198	173	127	99	62	30	0
149	155	143	133	73	33	12	0
67	62	59	34	19	0	0	0
172	132	105	91	60	0	0	0
61	46	37	14	0	0	0	0
29	25	24	21	10	0	0	0
164	175	162	131	126	67	36	0
44	37	33	29	18	0	0	0
127	121	116	70	74	27	0	0
182	147	140	118	96	24	11	0
28	21	17	8	0	0	0	0
149	161	146	118	121	64	34	0
117	131	118	106	67	23	0	0
95	94	81	60	20	0	0	0
56	41	34	13	0	0	0	0
174	117	84	40	0	0	0	0
39	33	28	22	7	0	0	0
93	115	103	74	67	30	20	0
135	138	119	80	78	39	22	0
48	35	33	11	0	0	0	0
197	197	197	173	89	22	0	0
113	113	111	77	78	28	15	0
67	60	52	27	15	0	0	0
81	78	74	38	28	0	0	0
168	198	180	148	119	62	30	0
144	141	125	128	120	59	36	0
56	46	45	11	0	0	0	0
75	70	65	29	23	0	0	0
72	69	58	41	19	0	0	0
159	190	162	134	91	45	21	0
41	37	37	26	12	0	0	0
137	134	134	81	26	0	0	0
53	42	38	17	11	0	0	0
38	24	21	14	11	0	0	0
200	200	194	146	132	62	36	0
42	33	26	8	0	0	0	0
67	58	34	36	17	0	0	0
157	135	133	80	25	0	0	0
94	89	76	66	55	27	15	0

Zusammenfassung

Die Auswertung von in vitro Tests zur Messung der Arzneimittelempfindlichkeit von Plasmodium falciparum

Die in vivo Prüfung der Arzneimittelempfindlichkeit von *Plasmodium falciparum* gestattet keine genaue, quantitative Ermittlung der Sensibilität des Parasiten. Dem gegenüber eignen sich in vitro Verfahren zur quantitativen Prüfung der Medikament-abhängigen Hemmung der Schizontenreifung. Ein einschlägiges, standardisiertes Prüfverfahren wird weltweit verwendet. Das Material unterliegt der Qualitätskontrolle. Das Verfahren eignet sich auch für periphere Laboratorien. Die Auswertung der Endpunkte der Schizontenreifung erlaubt einen raschen Überblick, kann eine gründliche quantitative Analyse jedoch nicht ersetzen. Aufgrund der log-normalen Verteilung der Arzneimittelempfindlichkeit in natürlichen Isolaten von *P. falciparum* erscheint hierfür ein auf den logarithmischen Konzentrationen und den Hemmprobiten basierendes Verfahren am besten geeignet. Ein zweckorientiertes EDV-Verfahren wurde entwickelt.

Schlüsselwörter

Plasmodium falciparum, Arzneimittelempfindlichkeit, Datenverarbeitung.

Table 4:

Computer output providing the key sensitivity parameters for chloroquine in 44 isolates of *Plasmodium falciparum* in an area of East Africa (E-group 2), 1994.

n	= 44	S	= 2.9034	f_S	= 1.2513
a	= 5.0025	A	= 1.3505	$f_{EC\ 50}$	= 1.2930
b	= 0.9330	K	= 7	$f_{EC\ 90}$	= 1.4749
r	= 0.9965	N'	= 132	$f_{EC\ 95}$	= 1.5690
Chi^2	= 0.7103	R	= 64	$f_{EC\ 99}$	= 1.7971

Drug. conc.	SMI %
0.2	7.72
0.4	17.67
0.8	44.13
1.6	64.56
3.2	88.54
6.4	95.07
12.8	100.00

EC	Mean	95% Confidence Intervals	
		Lower	Higher
EC_1	0.0824	0.0459	0.1481
EC_{16}	0.3435	0.2657	0.4441
EC_{50}	0.9973	0.7713	1.2896
EC_{84}	2.8957	2.2394	3.7442
EC_{90}	3.9390	2.6708	5.8095
EC_{95}	5.8142	3.7058	9.1223
EC_{99}	12.0689	6.7157	21.6891

Table 5:

Computer output providing the key sensitivity parameters for chloroquine in 41 isolates of *Plasmodium falciparum* in an area of East Africa (E-group 1), 1988.

n	= 41	S	= 2.2348	f_S	= 1.1668
a	= 6.0304	A	= 1.2278	$f_{EC\text{-}50}$	= 1.2224
b	= 1.2367	K	= 6	$f_{EC\text{-}90}$	= 1.3282
r	= 0.9960	N'	= 123	$f_{EC\ 95}$	= 1.3829
Chi^2	= 0.6302	R	= 32	$f_{EC\ 99}$	= 1.5134

Drug. conc.	SMI %
0.2	18.60
0.4	42.05
0.8	76.98
1.6	96.46
3.2	99.19
6.4	100.00
12.8	100.00

EC	Mean	95% Confidence Intervals	
		Lower	Higher
EC_1	0.0663	0.0438	0.1003
EC_{16}	0.1945	0.1591	0.2378
EC_{50}	0.4347	0.3556	0.5314
EC_{84}	0.9714	0.7946	1.1875
EC_{90}	1.2252	0.9225	1.6273
EC_{95}	1.6436	1.1885	2.2730
EC_{99}	2.8516	1.8843	4.3156

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