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EVALUATION OF IN VITRO TESTS FOR DRUG SENSITIVITY IN PLASMODIUM FALCIPARUM: PROBIT ANALYSIS OF LOGDOSE/RESPONSE TEST FROM 3-8 POINTS ASSAY

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1. INTRODUCTION

The evaluation of schizont maturation tests such as the in vitro macrotest and microtest poses certain difficulties. In particular the determination of essential parameters such as the drug concentration inhibiting schizont maturation at 50% or 90% level (i.e. EC_{50} or EC_{90}) requires cumbersome and complicated calculations as well as an acquaintance with the mathematical procedure. Some indications may be obtained from the cut-off point, i.e. the drug concentration which leads to a complete inhibition of schizont maturation, and from a graphic display of the observed data. However, this procedure is not satisfactory for a reliable estimate of the parameters of drug sensitivity.

Therefore a programme has been developed for the probit analysis of logdose/response tests from 3 to 8 points assays which, with the use of a Texas Instruments TI-59 calculator and its Applied Statistics Module, permits the simple processing of drug sensitivity data and yields all essential parameters. The programme is given in Annex 1.

The procedure is applicable to all types of schizont maturation tests (macro- and microtests), and to the results of individual tests as well as to grouped data. It is not meant for the statistical comparison of effective concentrations and other parameters (point studies in different areas or groups, or longitudinal investigations) which would require a different type of programme.

2. PREPARATION OF DATA FOR PROCESSING

The evaluation system is based on the number of plasmodia which, either in the controls or in the drug-treated specimen, have reached schizont stage. Three elements are required:

(a) The number of schizonts in the control (number of schizonts per 300 or 1000 leukocytes in the macrotest; number of schizonts per 200 asexual parasites in the microtest).

(b) The drug concentrations used in the test.

(c) The number of schizonts determined in the presence of the various drug concentrations (NB: the number of schizonts is used, not a % figure related to controls).

2.1 Individual tests

2.1.1 Macrotest

The data are taken from the WHO form 5407 "Response of P. falciparum to chloroquine and mefloquine (in vitro-test)" (see Annex 2) and are listed as follows:

Drug concentration (n-mol)	Control (mean of the two readings)	0.25	0.50	0.75	1.00	1.25	1.50	2.00	3.00
No. of schizonts	...	...	...	...	...	...	...	...	...

The concentration lines of chloroquine and mefloquine are identical. The concentrations correspond directly to x 10^{-6} mol/l blood.

2.1.2 Microtest

The data are taken from the WHO form 5407 "Response of P. falciparum to chloroquine and mefloquine (in vitro-test)" (see Annex 2) and are listed as follows:

(a) Chloroquine

Well	A	B	C	D	E	F	G	H
Drug dose p-mol per well	CONTROL	1	2	4	5.7	8	16	32
Drug concentration x 10^{-6} mol/l blood	CONTROL	0.20	0.40	0.80	1.14	1.60	3.20	6.40
No. of schizonts	...	...	...	...	...	...	...	...

(b) Mefloquine

Well	A	B	C	D	E	F	G	H
Drug dose p-mol per well	CONTROL	0.5	1.0	2.0	4.0	5.7	8.0	16.0
Drug concentration x 10^{-6} mol/l blood	CONTROL	0.10	0.20	0.40	0.80	1.14	1.60	3.20
No. of schizonts	...	...	...	...	...	...	...	...

These data are ready for immediate processing (see section 3 below), provided that the control readings of schizonts are higher than those obtained in any drug well or vial. Should this not be the case, the highest value is used in the place of the control.

Please note that the drug doses per well (microtest) have been given only for the purpose of orientation. Their use in data processing and graphic display has become obsolete. All processing in the context of this paper is effected with the drug concentration values (on the basis of 10^{-6} mol/l blood).

2.2 Grouped material

In principle, grouped data are sequentially listed in the same way as described above for the individual tests. At the end, the figures in each column are added up, as in the following example of 15 microtests for chloroquine sensitivity which were carried out, at the same time, in a village in eastern Asia.

	Number of schizonts per 200 asexual *P. falciparum*							
Well	A	B	C	D	E	F	G	H
Drug concentration x 10^{-6} mol/l blood	CONTROL	0.20	0.40	0.80	1.14	1.60	3.20	6.40
Case 1	87	76	65	-	41	35	16	7
Case 2	144	138	112	59	53	41	21	13
Case 3	92	-	66	-	45	27	12	6
Case 4	157	138	128	96	71	58	31	12
Case 5	63	59	47	36	32	23	9	0
Case 6	106	101	95	55	49	39	20	9
Case 7	98	87	69	61	44	28	14	0
Case 8	141	129	108	83	67	51	27	11
Case 9	138	124	93	72	58	42	26	12
Case 10	75	71	56	38	35	30	15	6
Case 11	87	-	71	54	39	31	11	1
Case 12	159	143	124	97	76	54	29	10
Case 13	58	54	49	33	20	18	0	0
Case 14	123	107	102	72	42	39	16	-
Case 15	149	119	113	87	66	57	21	10
TOTAL	1 677	1 346	1 298	843	738	573	268	97

It should be noted that some readings are missing (e.g. due to contamination of well or loss of thick film), such as the readings at concentration 0.8 x 10^{-6} mol/l in cases No. 1 and 3; therefore the total number of schizonts in the controls would be inexact if related to the total number of schizonts in the 0.8 x 10^{-6} mol/l concentration. In that event an appropriate adjustment needs to be made and the control values of cases with missing concentration readings should be excluded from the overall total number of schizonts in the controls as related to the particular concentration. A summary table of the grouped data is to be prepared as follows:

Drug concentration x 10^{-6} mol/l blood	0.20	0.40	0.80	1.14	1.60	3.20	6.40
Total number of schizonts counted in group	1 346	1 298	843	738	573	268	97
Total number of schizonts in controls (related to readable wells)	1 498	1 677	1 498	1 677	1 677	1 677	1 554

From this listing it is seen that the total number of schizonts in controls is not the same with regard to all concentrations since readings at 0.2 (x 10^{-6} mol/l) were missing in cases No. 3 and 11, those at 0.8 (x 10^{-6} mol/l) in cases No. 1 and 3, those at 6.4 (x 10^{-6} mol/l) in case No. 14.

Care should be taken to include only those tests which have produced a minimum of 20 schizonts in the controls. In the unlikely event that the total number of schizonts in the controls is inferior to the total number in a specific drug concentration group, the latter should be employed in the place of the "total number of schizonts in controls".

Macrotests and microtests are grouped in the same way. However, macrotests may produce a maximum of 8 data points (unless concentrations of >3.0 x 10^{-6} mol/l blood are employed), whereas the microtest produces not more than 7 data points.

3. DATA PROCESSING (TI-59)

3.1 Mode of operation

Insert the Applied Statistics Module.

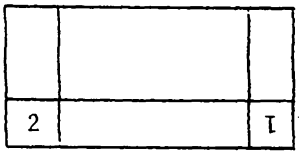
Switch on.

Press: 2nd CP.

"Read" first field of card 1 (let card pass through hole from right to left, black side down).

Press: CLR (clear).

"Read" second field of card 1 (same card, also from right to left, but card reversed, again black side down).



Press: CLR.

"Read" card 2 (same way as for the first field only of card 1).

(Each time a card has passed, the number of the card will be visible. If the display flashes off and on, repeat procedure. If this procedure fails, carefully clean the card, the machine reading head and drive roller.)

3.2 Entering of data

The programme accepts assays with a minimum of three points and a maximum of eight points.

Procedure (press keys in the indicated sequence):

No. of data point		Dose		No. of schizonts at data point		No. of schizonts in control	
1	A	....	R/S	.....	R/S	....	R/S
2							
.							
.							
.							
8	A	....	R/S	.....	R/S	....	R/S

Correct entering of data can be checked now by repeatedly pressing R/S; all the data become visible in sequence.

The point data can be entered in any sequence, but when the point code (from 1 to 8) and the label A have been pressed, the dose, response and total must be entered in this sequence. This allows the correction or the changing of the information relating to any point without starting again from the beginning. Note that all the three parameters must be entered even if only one of them needs to be modified.

Pressing R/S after having entered the parameters of any point will initiate the checking process always from point 1 onwards. This review can be stopped at any place and new data entered.

In the example under section 2.2 the following sequence would have to be entered:

No. of data point	Key	Drug concentration (10^{-6} mol/l)	Key	No. of schizonts at data point (r)	Key	No. of schizonts in control (n)	Key
1	A	0.2	R/S	1 346	R/S	1 498	R/S
2	A	0.4	R/S	1 298	R/S	1 677	R/S
3	A	0.8	R/S	843	R/S	1 498	R/S
4	A	1.14	R/S	738	R/S	1 677	R/S
5	A	1.6	R/S	573	R/S	1 677	R/S
6	A	3.2	R/S	268	R/S	1 677	R/S
7	A	6.4	R/S	97	R/S	1 554	R/S

The correct entering of the data can now be checked by pressing once again R/S (display 0.2). Subsequent pressing of R/S will show 1346; 1498; 0.4; 1298; etc. If one entry is found to be incorrect, this should be noted, but it will be useful to continue the checking until the very end of the data assembly, i.e. ... 6.4; 97; 1554. Then the faulty line in its totality needs to be keyed in, e.g. if it was line 4, the following sequence needs to be keyed in: [4] [A] 1.14 [R/S] 738 [R/S] 1677 [R/S]. This can be rechecked by repeatedly pressing [R/S] (this brings the whole sequence of data to the display, from the beginning). If all data are found to be correct, one can move to the steps described under section 3.3.

3.3 Display of observed inhibition of schizont maturation

Press [B]. The machine stops when the value 100 $(1-r/n)$% for point 1 (% maturation inhibition) is displayed. Press [R/S] successively to see the % values of schizont maturation inhibition observed at points 2, 3, 4, etc. Press [R/S] again after the last % displayed to start the computation which may last eight minutes. When it stops, the number of iterations completed is displayed. The iteration process stops when the difference between two successive estimates of the slope is equal to or less than 5% of the standard error of the last slope estimate. The programme allows for eight successive iterations.

Step [B] cannot be repeated as the data have been modified by this step.

In the example under section 2.2 the following readings can be taken after pressing [B]:

point 1	10.147 (%)	[R/S]
point 2	22.600 (%)	[R/S]
point 3	43.725 (%)	[R/S]
point 4	55.993 (%)	[R/S]
point 5	65.832 (%)	[R/S]
point 6	84.019 (%)	[R/S] approx. 8 min.
point 7	93.758 (%)	[R/S] - - - - - - ->

display shows the number of iterations (3).

3.4 Probit analysis

Press [C]: after approximately 20 seconds the χ^2 probability for heterogeneity will be displayed. If the value is less than 0.05 (indicating significant heterogeneity at 5% probability level), the confidence limits of the effective concentrations will be considerably underestimated and should therefore be ignored under section 3.5.

Press [R/S]: the display shows the inhibition (in probits) at concentration 1 (i.e. the probit for logdose = 0).

Press [R/S]: the display shows slope (b) of the probit regression line.

Press [R/S]: the display shows the variance of the slope (S_b^2), corrected for heterogeneity.

The above-shown probit analysis procedure can be repeated by pressing key [C] again.

In the example under section 2.2 the following results will be obtained after pressing [C]:

χ^2 probability for heterogeneity = 0.925106 (no significant heterogeneity)

Inhibition at concentration 1 : 5.027197 probits

Slope b : 1.892188

Variance of slope (S_b^2) : 0.001136 (corrected for heterogeneity)

3.5 Calculation of effective concentrations

For calculation of the effective concentrations (ECs) any % of theoretical response between 1% and 99% is entered, followed by pressing key [D]. For instance for EC_{50} (i.e. the drug concentration inhibiting schizont maturation at 50% level), 50 is entered followed by pressing [D]. The EC_{50} is then displayed; press key [R/S] for obtaining the lower confidence limit, and [R/S] again for obtaining the higher confidence limit (the lower and higher confidence limits should be disregarded if the χ^2 probability for heterogeneity is <0.05 - see section 3.4 above.

The operation can be repeated for any EC between EC_1 and EC_{99} (the calculation of the EC takes approximately 30-40 seconds).

In the example of section 2.2, one would proceed as follows for the calculation of EC_1, EC_5, EC_{10}, EC_{50}, EC_{90}, EC_{95} and EC_{99}.

Parameter required	Enter	Key	Display of EC	Key	Display of lower confidence limit	Key	Display of higher confidence limit
EC_1	1	[D]	0.0570	[R/S]	0.0511	[R/S]	0.0636
EC_5	5	[D]	0.1307	[R/S]	0.1205	[R/S]	0.1419
EC_{10}	10	[D]	0.2034	[R/S]	0.1901	[R/S]	0.2176
EC_{50}	50	[D]	0.9674	[R/S]	0.9361	[R/S]	0.9998
EC_{90}	90	[D]	4.6010	[R/S]	4.3270	[R/S]	4.8924
EC_{95}	95	[D]	7.1589	[R/S]	6.6403	[R/S]	7.7180
EC_{99}	99	[D]	16.4169	[R/S]	14.8157	[R/S]	18.1912

For the graphic display of the regression line one requires only two points. (For higher precision it is suggested that two fairly distant points be selected within the range of the log probit paper used, for instance in the above-mentioned example EC_5 and EC_{95}.) However, if the χ^2 probability for heterogeneity is >0.05 (no significant heterogeneity), as in the above example, one would calculate a wider range of ECs in order to obtain the lower and the higher confidence limits which should also be shown in the graph. The graphic display should also contain the observed data points (see section 3.3 and Fig. 1).[1]

3.6 Resetting of calculating machine

If data of another assay are to be processed, press [2nd] [CMs] and proceed as from section 3.2 with the data entry (there is no need to read the programme cards again as long as the machine has not been switched off).

3.7 Additional example of logdose/response analysis

The following example is based on tests conducted in Burma,[2] using the results of 16 *in vitro* microtests which are all related to R-I *in vivo* responses.

3.7.1 Grouping of material

The following tests were grouped:

				Number of schizonts				
Well	A	B	C	D	E	F	G	H
CHLOROQUINE x 10^{-6} mol/l blood	CONTROL	0.20	0.40	0.80	1.14	1.60	3.20	6.40
Case No. 25	96	92	80	80	80	76	60	52
Case No. 26	60	60	42	30	30	24	12	12
Case No. 27	58	58	58	50	16	12	6	6
Case No. 28	120	120	104	68	-	70	-	40
Case No. 29	50	48	48	42	36	32	12	0
Case No. 31	86	80	74	70	62	58	22	8
Case No. 33	62	52	44	36	28	16	0	0
Case No. 34	92	80	66	40	44	40	40	36
Case No. 35	60	48	54	42	16	18	6	4
Case No. 37	72	60	44	32	24	20	24	16
Case No. 40	80	80	68	60	52	40	16	10
Case No. 41	96	72	68	56	56	48	32	24
Case No. 42	108	108	100	94	90	84	72	62
Case No. 43	72	64	58	52	48	36	12	6
Case No. 44	58	52	44	36	32	26	16	6
Case No. 45	24	24	20	16	16	12	4	0
	1 194	1 098	972	804	630	612	334	282

[1] The regression line in Fig. 1 shows an EC_{95} higher than 1.0 x 10^{-6} mol chloroquine/l blood and is thus indicative of resistance.

[2] Myint-Lwin, Min-Zaw & Rooney, W. (1982) Comparative study of the micro *in vitro* and the *in vivo* tests of the response of *Plasmodium falciparum* to chloroquine in Burma (Unpublished document WHO/MAL/82.982).

3.7.2 Summary of grouped data

Chloroquine concentration (x 10^{-6} mol/l blood)	0.20	0.40	0.80	1.14	1.60	3.20	6.40
Total number of schizonts counted in group	1 098	972	804	630	612	334	282
Total number of schizonts in controls (related to readable wells)	1 194	1 194	1 194	1 074	1 194	1 074	1 194

3.7.3 Entering of data

After entering the programme into the machine (see section 3.1), the summary of the grouped data is keyed into the machine as follows:

No. of data point	Key	Drug concentration (x 10^{-6} mol/l)	Key	No. of schizonts at data point (r)	Key	No. of schizonts in control (n)	Key
1	A	0.2	R/S	1 098	R/S	1 194	R/S
2	A	0.4	R/S	972	R/S	1 194	R/S
3	A	0.8	R/S	804	R/S	1 194	R/S
4	A	1.14	R/S	630	R/S	1 074	R/S
5	A	1.6	R/S	612	R/S	1 194	R/S
6	A	3.2	R/S	334	R/S	1 074	R/S
7	A	6.4	R/S	282	R/S	1 194	R/S

3.7.4 Display of observed inhibition of schizont maturation

After pressing of [B] the observed inhibition (%) at data point 1 is displayed $\lfloor 100(1-r/n) \rfloor$. Successive pressing of key [R/S] yields the % inhibition values for data points 2-7, and the following readings are obtained:

Data point 1	Inhibition	8.040%
Data point 2	Inhibition	18.593%
Data point 3	Inhibition	32.663%
Data point 4	Inhibition	41.341%
Data point 5	Inhibition	48.744%
Data point 6	Inhibition	68.901%
Data point 7	Inhibition	76.382%

Pressing of [R/S] after the display of data point 7 initiates the probit analysis and approximately eight minutes later the display lights up again showing the figure 3, indicating that three iterations were effected to fit the regression to the observed data.

3.7.5 Probit analysis

After pressing [C] the value of χ^2 probability for heterogeneity is shown as 0.006893. This value is <0.05 and therefore the lower and higher confidence limits of the ECs are to be neglected (as they would be considerably underestimated).

Repeated pressing of [R/S] yields the following parameters:

Inhibition at concentration 1: 4.673711 probits

Slope b : 1.426642

Variance of the slope (S_b^2) : 0.003862 (corrected for heterogeneity)

3.7.6 Effective concentrations

Since the χ^2 probability for heterogeneity is <0.05 there is no point in obtaining the lower and higher confidence limits for the various effective concentrations EC_1, EC_5, EC_{10}, EC_{50}, EC_{90}, EC_{95} and EC_{99} are obtained by entering the appropriate figure, followed by pressing the key [D]:

For EC	Enter	Key	Display (rounded figures)
EC_1	1	[D]	0.0396
EC_5	5	[D]	0.1191
EC_{10}	10	[D]	0.2140
EC_{50}	50	[D]	1.6932
EC_{90}	90	[D]	13.3948
EC_{95}	95	[D]	24.0756
EC_{99}	99	[D]	72.3843

3.7.7 Graphic display

The resulting regression and the observed data points are shown in Fig. 2. For plotting the regression only two ECs (e.g. EC_5 and EC_{95}) would have been sufficient. The graph shows a rather good fit of the regression line to the observed data points, especially in the lower concentration ranges.

4. PROGRAMME

The probit analysis is based on a programme using 560 steps. This programme, listed in Annex 1, needs to be manually entered in the TI-59 either for execution or to keep it on the magnetic cards required for running further analyses (see section 3.1 above).

For this purpose the Applied Statistics Module is inserted in the calculator.

After switching the calculator on, key in 5 [2nd] [Op] 17 (display 559.49) to change the partition as required; then press key [LRN] and enter the programme listed in Annex 1 step by step (do not forget to use prefix [2nd] for all keys requiring it, e.g. [Prd], [Exc], [St flg]). Once the programme is entered press key LRN (this switches the machine from the learn mode to the keyboard control).

Before writing the programme on magnetic cards it is advisable to re-establish the original memory partition by keying in:

6 [2nd] [Op] 17 (display: 479.59);

This will permit the reading of the magnetic cards without modifying the partition, as the necessary change is made by the programme itself in the course of execution.

The programme can then be recorded on magnetic cards (two will be required for this purpose), as follows:

(a) Press 1 [2nd] [Write] and insert blank magnetic card (printed side up) into the lower slot of the machine (right side). The card comes out on the left side, carrying now the programme of the first data bank. Write the figure 1 in the left upper corner of the magnetic card.

(b) Press 2 [2nd] [Write] and insert same card, printed side up, but label upside down (black heading down), into the lower slot of the machine (right side). Write the figure 2 in the right upper corner of the magnetic card.

(c) Press 3 [2nd] [Write] and insert another blank magnetic card (printed side up) and proceed as above. Write the figure 3 in the left upper corner of the magnetic card.

It is useful to write a programme title on the magnetic cards for easy identification (e.g. Logdose/response analysis, 3-8 points, Drug sensitivity P. falciparum).

The machine can now be used for data analysis as described in sections 3.2-3.6; once the calculator has been switched off, the programme will vanish and it needs to be entered again manually or from the magnetic cards (see section 3.1) before proceeding with a new analysis.

NB: copies of magnetic cards loaded with the above-mentioned programme can be obtained from:

World Health Organization
Attention: Chief MAP/RTI
Avenue Appia
CH-1211 Geneva 27
Switzerland

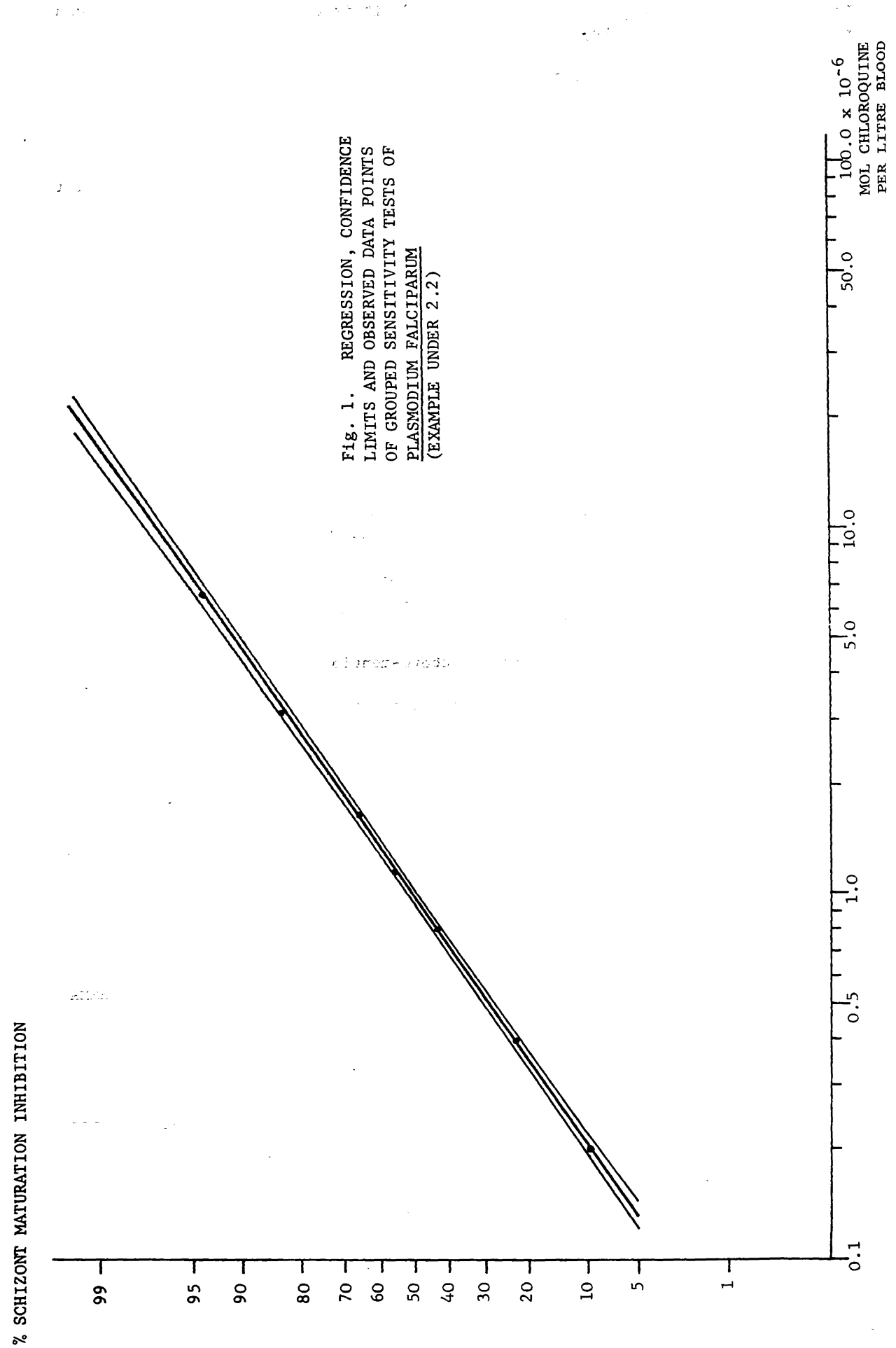


Fig. 1. REGRESSION, CONFIDENCE LIMITS AND OBSERVED DATA POINTS OF GROUPED SENSITIVITY TESTS OF PLASMODIUM FALCIPARUM (EXAMPLE UNDER 2.2)

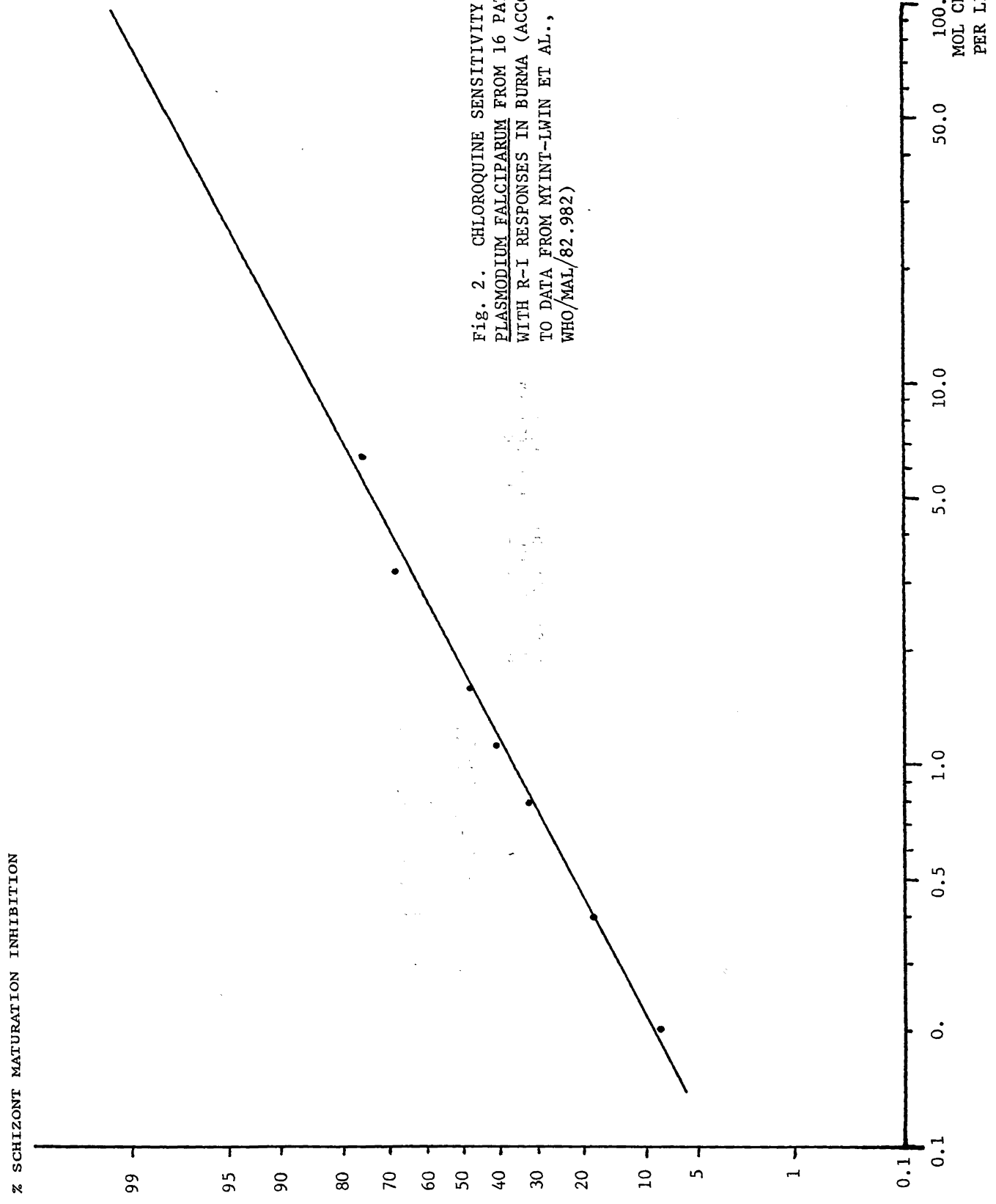


Fig. 2. CHLOROQUINE SENSITIVITY OF PLASMODIUM FALCIPARUM FROM 16 PATIENTS WITH R-I RESPONSES IN BURMA (ACCORDING TO DATA FROM MYINT-LWIN ET AL., WHO/MAL/82.982)

ANNEX 1

PROGRAMME

000	76	LBL
001	11	A
002	65	×
003	05	5
004	85	+
005	02	2
006	95	=
007	42	STO
008	00	00
009	03	3
010	42	STO
011	01	01
012	91	R/S
013	72	ST*
014	00	00
015	01	1
016	44	SUM
017	00	00
018	97	DSZ
019	01	01
020	00	00
021	12	12
022	04	4
023	42	STO
024	00	00
025	02	2
026	44	SUM
027	00	00
028	03	3
029	42	STO
030	01	01
031	91	R/S
032	01	1
033	44	SUM
034	00	00
035	73	RC*
036	00	00
037	97	DSZ
038	01	01
039	00	00
040	31	31
041	91	R/S
042	02	2
043	44	SUM
044	00	00
045	03	3
046	42	STO
047	01	01
048	61	GTO
049	00	00
050	32	32
051	76	LBL
052	12	B
053	29	CP
054	00	0
055	42	STO
056	03	03
057	42	STO
058	49	49
059	04	4
060	42	STO
061	00	00
062	05	5
063	44	SUM
064	00	00
065	73	RC*
066	00	00
067	67	EQ
068	00	00
069	76	76
070	01	1
071	44	SUM
072	03	03
073	61	GTO
074	00	00
075	62	62
076	43	RCL
077	03	03
078	42	STO
079	01	01
080	02	2
081	42	STO
082	31	31
083	03	3
084	42	STO
085	35	35
086	04	4
087	42	STO
088	36	36
089	05	5
090	44	SUM
091	31	31
092	44	SUM
093	35	35
094	44	SUM
095	36	36
096	01	1
097	75	-
098	73	RC*
099	35	35
100	55	÷
101	73	RC*
102	36	36
103	95	=
104	72	ST*
105	35	35
106	65	×
107	01	1
108	00	0
109	00	0
110	95	=
111	91	R/S
112	73	RC*
113	31	31
114	28	LOG
115	72	ST*
116	31	31
117	97	DSZ
118	01	01
119	00	00
120	89	89

Annex 1

```
121  05  5
122  69 □P
123  17  17
124  00  0
125  42 STO
126  30  30
127  01  1
128  42 STO
129  06  06
130  08  8
131  42 STO
132  02  02
133  22 INV
134  86 STF
135  01  01
136  43 RCL
137  03  03
138  42 STO
139  01  01
140  00  0
141  42 STO
142  31  31
143  42 STO
144  35  35
145  42 STO
146  36  36
147  42 STO
148  40  40
149  42 STO
150  41  41
151  42 STO
152  45  45
153  02  2
154  42 STO
155  47  47
156  03  3
157  42 STO
158  48  48
159  04  4
160  42 STO
161  49  49
162  05  5
163  44 SUM
164  47  47
165  44 SUM
166  48  48
167  44 SUM
168  49  49
169  87 IFF
170  01  01
171  01  01
172  78  78
173  71 SBR
174  24 CE
175  61 GTO
176  02  02
177  26  26
178  43 RCL
179  04  04
180  85  +
181  43 RCL
182  30  30
183  65  ×
184  73 RC*
185  47  47
186  95  =
187  42 STO
188  10  10
189  36 PGM
190  19  19
191  12  B
192  85  +
193  32 X↕T
194  95  =
195  43 RCL
196  10  10
197  36 PGM
198  19  19
199  11  A
200  42 STO
201  06  06
202  49 PRD
203  06  06
204  32 X↕T
205  22 INV
206  49 PRD
207  06  06
208  94 +/-
209  85  +
210  01  1
211  95  =
212  22 INV
213  49 PRD
214  06  06
215  75  -
216  01  1
217  85  +
218  73 RC*
219  48  48
220  95  =
221  55  ÷
222  32 X↕T
223  95  =
224  44 SUM
225  10  10
226  73 RC*
227  49  49
228  65  ×
229  43 RCL
230  06  06
231  95  =
232  42 STO
233  25  25
234  44 SUM
235  31  31
236  65  ×
237  73 RC*
238  47  47
239  95  =
240  44 SUM
241  35  35
242  65  ×
243  73 RC*
244  47  47
245  95  =
246  44 SUM
247  36  36
248  43 RCL
249  25  25
250  65  ×
251  43 RCL
252  10  10
253  95  =
254  44 SUM
255  40  40
256  65  ×
257  43 RCL
258  10  10
259  95  =
260  44 SUM
261  41  41
262  55  ÷
263  43 RCL
264  10  10
265  65  ×
266  73 RC*
267  47  47
268  95  =
269  44 SUM
270  45  45
```

Annex 1

```
271  97 DSZ
272  01  01
273  01  01
274  62  62
275  43 RCL
276  35  35
277  33 X²
278  55  ÷
279  43 RCL
280  31  31
281  95  =
282  22 INV
283  44 SUM
284  36  36
285  43 RCL
286  40  40
287  33 X²
288  55  ÷
289  43 RCL
290  31  31
291  95  =
292  22 INV
293  44 SUM
294  41  41
295  43 RCL
296  35  35
297  65  ×
298  43 RCL
299  40  40
300  55  ÷
301  43 RCL
302  31  31
303  95  =
304  22 INV
305  44 SUM
306  45  45
307  43 RCL
308  31  31
309  22 INV
310  49 PRD
311  35  35
312  43 RCL
313  31  31
314  22 INV
315  49 PRD
316  40  40
317  43 RCL
318  45  45
319  55  ÷
320  43 RCL
321  36  36
322  95  =
323  42 STO
324  25  25
325  65  ×
326  43 RCL
327  35  35
328  75  -
329  43 RCL
330  40  40
331  95  =
332  94 +/-
333  42 STO
334  04  04
335  43 RCL
336  41  41
337  75  -
338  43 RCL
339  45  45
340  33 X²
341  55  ÷
342  43 RCL
343  36  36
344  95  =
345  42 STO
346  11  11
347  86 STF
348  01  01
349  43 RCL
350  36  36
351  35 1/X
352  34 ΓX
353  65  ×
354  93  .
355  00  0
356  05  5
357  95  =
358  32 X:T
359  43 RCL
360  25  25
361  75  -
362  48 EXC
363  30  30
364  95  =
365  50 I×I
366  77  GE
367  03  03
368  72  72
369  61 GTO
370  03  03
371  76  76
372  97 DSZ
373  02  02
374  01  01
375  36  36
376  09  9
377  75  -
378  43 RCL
379  02  02
380  95  =
381  91 R/S
382  76 LBL
383  24 CE
384  01  1
385  02  2
386  42 STO
387  00  00
388  01  1
389  93  .
390  02  2
391  42 STO
392  46  46
393  00  0
394  42 STO
395  10  10
396  43 RCL
397  10  10
398  36 PGM
399  19  19
400  12  B
401  75  -
402  73 RC*
403  48  48
404  95  =
405  94 +/-
406  69 OP
407  10  10
408  65  ×
409  43 RCL
410  46  46
411  95  =
412  44 SUM
413  10  10
414  93  .
415  05  5
416  49 PRD
417  46  46
418  97 DSZ
419  00  00
420  03  03
```

Annex 1

```
421  96  96
422  92 RTN
423  76 LBL
424  13  C
425  43 RCL
426  03  03
427  75  -
428  02  2
429  95  =
430  42 STO
431  41  41
432  55  ÷
433  43 RCL
434  11  11
435  95  =
436  35 1/X
437  42 STO
438  45  45
439  43 RCL
440  41  41
441  36 PGM
442  21  21
443  11  A
444  43 RCL
445  11  11
446  36 PGM
447  21  21
448  13  C
449  75  -
450  01  1
451  95  =
452  94 +/-
453  91 R/S
454  32 X:T
455  93  .
456  00  0
457  05  5
458  77  GE
459  04  04
460  64  64
461  01  1
462  42 STO
463  45  45
464  43 RCL
465  04  04
466  85  +
467  05  5
468  95  =
469  91 R/S
470  43 RCL
```

```
471  30  30
472  91 R/S
473  43 RCL
474  45  45
475  55  ÷
476  43 RCL
477  36  36
478  95  =
479  91 R/S
480  76 LBL
481  14  D
482  65  ×
483  93  .
484  00  0
485  01  1
486  95  =
487  72 ST*
488  48  48
489  00  0
490  42 STO
491  10  10
492  73 RC*
493  48  48
494  32 X:T
495  93  .
496  05  5
497  67  EQ
498  05  05
499  02  02
500  71 SBR
501  24 CE
502  43 RCL
503  10  10
504  75  -
505  43 RCL
506  04  04
507  95  =
508  55  ÷
509  43 RCL
510  30  30
511  95  =
512  42 STO
513  46  46
514  22 INV
515  28 LOG
516  95  =
517  91 R/S
518  43 RCL
519  46  46
520  75  -
```

```
521  43 RCL
522  35  35
523  95  =
524  33 X²
525  55  ÷
526  43 RCL
527  36  36
528  85  +
529  43 RCL
530  31  31
531  35 1/X
532  95  =
533  34 ΓX
534  55  ÷
535  43 RCL
536  30  30
537  65  ×
538  02  2
539  95  =
540  94 +/-
541  85  +
542  43 RCL
543  46  46
544  95  =
545  22 INV
546  28 LOG
547  91 R/S
548  28 LOG
549  94 +/-
550  85  +
551  02  2
552  65  ×
553  43 RCL
554  46  46
555  95  =
556  22 INV
557  28 LOG
558  91 R/S
559  00  0
```

ANNEX 2

RESPONSE OF P. FALCIPARUM TO CHLOROQUINE AND MEFLOQUINE (IN VITRO-TEST)

A COUNTRY AND PLACE OF TEST

No. _____ Serial No.: 1

Institution _____ City/Town _____ Country Code: 5

Investigator _____ Country _____ Institution: 8

Province/State _____ District/County _____

B COUNTRY AND PLACE INFECTION PROBABLY CONTRACTED

Country _____ Country Code: 10 Prov Code:

Province/State _____ Lat. in box 15 1 = N 2 = S 15 deg min

District/County _____ Locality _____ Long. in box 20 1 = E 2 = W 20 deg min

C DATE AND TIME BLOOD TAKEN

_____ 26 day month year hour min

D INCUBATION TIME

Date _____ Duration (hours) _____ Started: 36 hour min Terminated hour min

E PATIENT

Sex: M F Less than 1 year = 00 Age: 44 years

F REASON FOR SCREENING

1 = Resistant or suspected resist. case

2 = Collateral case of resist. or suspect. resist. case

3 = Resist. in area of origin

4 = Resist. in area of orig. (abroad)

5 = Resist. in adjacent area

6 = Resist. in other rel. area

7 = Routine monitoring

8 = Other _____ 46

G SAMPLE

1 = General pop. 2 = School 3 = Labour force 4 = Outpatient 5 = Inpatient 6 = Migrant labour 7 = Other 47

H DRUG TAKEN DURING LAST 2 WEEKS

HISTORY: Any antimal. drug taken? 1 = Yes 2 = No 3 = ? (box 48) 48

If "Yes" specify drug(s): _____ (box 49) 49

URINE-TEST: 1 = pos 2 = neg 3 = Doubtful 4 = Not done

4-aminoquinolines (box 50) 50

Sulfanomides (box 51) 51

I PRE-CULTURE SLIDE-EXAM.

No. asexual P.f. per mm³ blood: _____

ASEXUAL P. FALCIPARUM

No. counted: small _____ medium _____ large _____

total 52 WBC counted

J RESULT OF MACRO-TEST

Chloroquine Kit batch No.: 61 Mefloquine Kit batch No.: 63

CHLOROQUINE n mol/vial →

1 SCHIZONT./300 leuc.

2 SCHIZONT./1000 leuc 65 → 66

Control 1	Control 2	0.25	0.50	0.75	1.00	1.25	1.50	2.00	3.00
Mean K: _____		_____%	_____%	_____%	_____%	_____%	_____%	_____%	_____%

MEFLOQUINE n mol/vial →

1 SCHIZONT./300leuc.

2 SCHIZONT./1000 leuc 96 → 97

Control 1	Control 2	0.25	0.50	0.75	1.00	1.25	1.50	2.00	3.00
Mean K: _____		_____%	_____%	_____%	_____%	_____%	_____%	_____%	_____%

K RESULT OF MICRO-TEST

Chloroquine plate batch No.: 127 Mefloquine plate batch No.: 129

CHLOROQUINE p mol/well → SCHIZONT./200 paras → 131

Control	1	2	4	5.7	8	16	32
	_____%	_____%	_____%	_____%	_____%	_____%	_____%

MEFLOQUINE p mol/well → SCHIZONT./200 paras → 155

Control	0.5	1	2	4	5.7	8	16
	_____%	_____%	_____%	_____%	_____%	_____%	_____%

Average control: _____

L Were the slides referred for checking? 1 = yes 2 = no 179

M Has the patient travelled and where (during the last 12 months)? 180

N Conclusion: