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Evaluation of *Plasmodium cynomolgi* B Antigen in Enzyme Linked Immunosorbent Assay (ELISA) Test for Human Malaria

G.P. DUTTA[1], I.K. SRIVASTAVA[1], PAWAN SHARMA[1] and S.S. AGARWAL[2]

Antigen prepared from *Plasmodium cynomolgi* B schizonts was evaluated in the enzyme linked immunosorbent assay (ELISA) for the seroepidemiology of human malaria, using sera from malaria cases residing in seven different localities. At a cut off point equivalent to mean + 3 S D (Extinction at 492 nm. E_{492} = 0.066) of the values obtained in the normal healthy subjects (n = 155), seropositivity of 96.30, 1.43 and 11.11% was obtained in the slide positive malaria patients (n = 267), patients of pyrexia (n = 70) and the random hospital patients (n = 135), respectively. None of the normal healthy subjects gave a positive ELISA reaction. Highest E_{492} values were also obtained among the malaria patients (mean ± S D = 0.120 ± 0.03 $P<0.001$). Seropositivity among *P. falciparum* cases (n = 171) was 94.83%, among *P. vivax* cases (n = 93) 95.70% while all the 3 cases with mixed infection gave positive ELISA reaction.
Sensitivity of the *P. cynomolgi* B antigen compared favourably with that of *P. falciparum* and *P. knowlesi* antigens; its specificity was found to be better than that of *P. knowlesi* antigen which gave 12 false positive results in contrast to 5 obtained with *P. cynomolgi* B antigen from among the patients of pyrexia and random hospital patients.

INTRODUCTION

Serological tests for the detection of specific antibodies have been shown to be valuable in the diagnosis of clinical cases of malaria as well as in the seroepidemiological studies (Meuwissen, 1974; Manawadu and Voller, 1978; Lobel *et al.*, 1976; Kagan, 1972). Compared to indirect haemagglutination (IHA) and indirect fluorescent antibody (IFA) tests which have been used earlier for seroepidemiology of human malaria, the recently developed enzyme linked immunosorbent assay (ELISA) test has been found to be highly specific for the detection of malaria antibodies and it is next to radioimmunoassay in its sensitivity (Voller *et al.*, 1977). Using *Plasmodium falciparum* antigen, the ELISA test has been successfully applied for delineating areas of high or low endemicity, for monitoring the rate of transmission of the disease and for evaluating the success of malaria control measures (Voller *et al.*, 1976, 1980; Quakyi, 1979; Edrissian *et al.*, 1979; Dutta *et al.*, 1982).

In India, however, the application of this assay for the seroepidemiological studies of human malaria has been very limited because of the

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[1]Division of Microbiology
Central Drug Research Institute, Lucknow-226001, India.
[2]Department of Medicine
K.G. Medical College, Lucknow-226001, India.

non-availability of highly purified homologous antigen (*P. falciparum*) in sufficient quantity (Mahajan *et al.*, 1981; Francis *et al.*, 1982). A task force of the Indian Council of Medical Research suggested the possible use and evaluation of simian malaria parasites viz. *P. knowlesi*, *P. cynomolgi*, *P. coatneyi* etc., as the alternative antigens for seroepidemiological studies on malaria. Our earlier studies have shown that the antigens prepared from *P. knowlesi* and *P. cynomolgi* are quite suitable for the IHA and IFA tests (Agarwal *et al.*, 1981, 1982, 1983). Antigen prepared from *P. knowlesi* was found suitable for the ELISA test as well (Srivastava *et al.*, 1983).

This is the first report on the evaluation of *P. cynomolgi* B antigen in the ELISA test for sero-epidemiology of human malaria; sensitivity of this antigen has been compared with our earlier results on *P. falciparum* and *P. knowlesi* antigens.

MATERIAL AND METHODS

Blood samples for sera of 267 malaria patients (Group I) residing in 7 different localities of Uttar Pradesh and Haryana were collected at the time of their slide preparation (Table 1). Of these, 171 were found to harbour *Plasmodium falciparum*, 93 *P. vivax* and the remaining 3 had a mixed infection with both the species. Sera from 70 patients of pyrexia of varied aetiology (Group II), 135 random hospital patients (Group III) and 155 normal healthy medical students (Group IV) were also collected. Subjects included in Group II and III were slide negative for malaria but the possibility of malaria infection in the past was not ruled out. Subjects included in Group IV were drawn from the medical students admitted to the King George's Medical College, Lucknow; special care was taken to include in this Group only those persons who had no history of malaria, presented no clinical signs or symptoms attributable to malaria and were slide negative. All sera were stored frozen at -20°C and inactivated at 56°C for 30 mins prior to use.

Antigen of *P. cynomolgi* B was prepared essentially according to the methods described earlier (Agarwal *et al.*, 1981; Srivastava *et al.*, 1983). Schizont infected erythrocytes from infected monkey blood were separated by

Table 1. Locality-wise break up of the malaria patients (Group 1)

Locality	No. of patients	No. with infection		
		P. falciparum	*P. vivax*	Mixed
1. Kalan (Shahjahanpur, U.P.)	55	43	9	3
2. Shaktinagar (Mirzapur, U.P.)	30	9	21	Nil
3. Ral (Mathura, U.P.)	41	36	5	Nil
4. Mant (Mathura, U.P.)	84	71	13	Nil
5. Purwa (Unnao, U.P.)	18	10	8	Nil
6. Chaurmastpur (Ambala, Haryana)	19	1	18	Nil
7. Karnal city hospital (Haryana)	20	1	19	Nil
Total	267	171	93	3

density gradient centrifugation. Schizonts obtained after saponin lysis were washed free of red cell ghosts and stored at -196°C in liquid nitrogen. On the day of test, an aliquot of the frozen schizonts was thawed, disrupted by ultrasonication and its soluble fraction used as antigen.

The optimal antigen concentration for coating the wells of the micro-ELISA plate (Cooke Microtitre, No. 1-223-29) was determined by a chequer board titration, using a reference positive and a reference negative serum.

The test was performed essentially according to Dutta *et al.* (1982) except that the antigen used was *P. cynomolgi*. The micro-ELISA plates were coated with antigen by airdrying 200 µl of the optimally diluted antigen in each of the wells. The antigen coated wells were washed with Tween saline solution and incubated with optimally diluted serum sample. This was followed by incubation with the optimally diluted enzyme conjugate viz. horse-radish peroxidase (HRP) labelled anti-human IgG (Cappel Labs., Chochranville, USA). Enzyme activity adhering to the wells was estimated biochemically using *o*-phenylenediamine dihydrochloride and H_2O_2 as substrate; the reaction was stopped by adding 5N H_2SO_4 and results recorded as extinction at 492 nm (E_{492}) after appropriate dilution of the sample. The WHO protocol was used for determination of optimal dilution of enzyme conjugate.

ELISA test using *P. knowlesi* and *P. falciparum* antigens was also performed on these sera as published elsewhere (Dutta *et al.*, 1982; Srivastava *et al.*, 1983) and the summary of results incorporated in the present paper for comparison.

The results were analysed using Student's 't' test.

RESULTS

Results of a chequer board titration for determining optimal dilutions of antigen and serum samples, presented in Table 2, showed that 1 in 100 dilution of the antigen as well as of the serum was optimal since the maximum difference in the E_{492} values of the positive (P) and negative (N) sera was observed at this dilution (P/N = 4).

In another chequer board titration results of which are shown in Table 3, a dilution of 1 in 1000 of the enzyme conjugate was found to be optimal for obtaining a clear cut distinction between positive and negative sera (P/N = 4).

Results obtained by the ELISA test among various groups of subjects are presented in Table 4. The highest E_{492} values were obtained among the malaria patients (Group I, $E_{492} = 0.1200 \pm 0.030$) and the lowest among the normal healthy subjects (Group IV, 0.030 ± 0.012). Taking mean + 2 SD of the values obtained in Group IV as the cut off point ($E_{492} = 0.0540$), 97.38% of the malaria patients (Group I), 10% of the patients with pyrexia of varied origin (Group II), 16.3% of the random hospital patients (Group III) and only 3.23% of the normal healthy subjects (Group IV) were found to be seropositive for malaria. Even at the cut off point equivalent to mean + 3SD of the normal values ($E_{492} = 0.066$) positivity obtained among malaria patients (96.3%) was largely unaffected while only 1 out of 70 patients (1.43%) of Group II and 15 out of 135 patients (11.11%) of Group III yielded a positive ELISA result; none of the healthy subjects gave a positive reaction at this cut off point.

Positivity of 96.55, 95.70 and 100% was obtained in cases of *P. falciparum*, *P. vivax* and mixed infection, respectively, at cut off point of Mean + 2SD. The corresponding positivity at mean + 3SD was 94.83, 95.7 and 100%, respectively.

Results of a comparative evaluation of the positivity obtained with *P. falciparum*, *P. knowlesi* and *P. cynomolgi* antigens in the ELISA test are presented in Table 5. Out of 267 malaria patients, 256 gave a positive result with

Table 2. Determination of optimal dilutions of antigen and serum using air dried *P.cynomolgi* B antigen coated plates

Antigen dilutions	Serum dilutions											
	1 : 50		1 : 100		1 : 200		1 : 400		1 : 800		1 : 1600	
	P	N	P	N	P	N	P	N	P	N	P	N
1 : 50	0.20	0.06	0.18	0.05	0.13	0.05	0.11	0.035	0.08	0.03	0.06	0.025
	(3.33)		(3.60)		(3.25)		(3.143)		(2.66)		(2.40)	
1 : 100	0.17	0.05	0.14	0.035	0.12	0.035	0.10	0.030	0.07	0.025	0.06	0.02
	(3.40)		(4.00)		(3.43)		(3.33)		(2.80)		(3.00)	
1 : 200	0.13	0.04	0.11	0.35	0.08	0.25	0.06	0.025	0.04	0.02	0.04	0.02
	(3.25)		(3.143)		(3.20)		(2.40)		(2.00)		(2.00)	
1 : 400	0.10	0.03	0.08	0.025	0.07	0.025	0.05	0.02	0.04	0.02	0.04	0.02
	(3.33)		(3.20)		(2.80)		(2.50)		(2.00)		(2.00)	

Control values -

No antigen = 0.015
No serum = 0.015
No conjugate = 0.005
Only substrate = 0.005

P - Reference positive serum
N - Reference negative serum

Figures in parentheses indicate P:N ratio.

Table 3. Standardization of optimum conjugate dilution for use in ELISA test using *P. cynomolgi* B antigen coated dry plates

Conjugate dilutions	E_{492} values of Ref. (+)ve serum	E_{492} values of Ref. (-)ve serum	Positive negative ratio (P/N)
1 : 250	0.24	0.07	3.43
1 : 500	0.20	0.055	3.64
1 : 1000	0.14	0.035	4.00
1 : 2000	0.10	0.030	3.66
1 : 4000	0.08	0.025	3.25

Table 4. Mean extinction values (E_{492}) among various groups and number of positive cases at different cut off points determined from normal values using *P. cynomolgi* B antigen

Groups	E_{492} values Mean ± S D	Positivity at		
		Mean + SD* (E_{492}=0.0420)	Mean + 2 SD* (E_{492}=0.0540)	Mean + 3 SD* (E_{492}=0.0660)
I. Malaria patients (n = 267)	0.1200±0.030 ($p<0.001$)*	264 (98.88)	260 (97.38)	257 (96.30)
(a) *P. falciparum* cases (n = 171)	0.1100±0.030 ($p<0.001$)*	169 (97.12)	168 (96.55)	165 (94.83)
(b) *P. vivax* cases (n = 93)	0.1200±0.040 ($p<0.001$)*	92 (98.93)	89 (95.70)	89 (95.70)
(c) Mixed infection cases (n = 3)	0.1400±0.020 ($p<0.001$)	3 (100.00)	3 (100.00)	3 (100.00)
II. Patients of pyrexia of varied origin (n = 70)	0.0400±0.009 (n.s.)*	15 (21.43)	7 (10.00)	1 (1.43)
III. Random hospital patients (n = 135)	0.0400±0.015 (n.s.)*	42 (31.11)	22 (16.30)	15 (11.11)
IV. Normal healthy subjects (n = 155)	0.0300±0.012	19 (12.26)	5 (3.23)	Nil

*These values are based on extinction values of normal healthy subjects (Group IV)
Figures in parentheses indicate percentage; n.s. = not significant; SD = Standard deviation.

Table 5. Comparative evaluation of positivity obtained with *P. falciparum, *P. knowlesi*** and *P. cynomolgi* B antigens in ELISA test using cut off point equal to mean + 3 SD of normal values (Group IV)**

Groups		No. of cases							
		Pf+ Pk+ Pc+	Pf- Pk- Pc-	Pf+ Pk+ Pc-	Pf+ Pk- Pc+	Pf+ Pk- Pc-	Pf- Pk+ Pc+	Pf- Pk- Pc+	Pf- Pk+ Pc-
I.	Malaria patients (n = 267)	256 (95.88)	Nil	8 (2.99)	Nil	2 (0.74)	Nil	1 (0.37)	Nil
(a)	*P. falciparum* cases (n = 171)	165 (96.49)	Nil	5 (2.92)	Nil	1 (0.58)	Nil	Nil	Nil
(b)	*P. vivax* cases (n = 93)	88 (94.62)	Nil	3 (3.22)	Nil	1 (1.08)	Nil	1 (1.08)	Nil
(c)	Mixed infection cases (n = 3)	3 (100)	Nil	Nil	Nil	Nil	Nil	Nil	Nil
II.	Patients of pyrexia of varied aetiology (n = 66)	Nil	60 (90.91)	Nil	Nil	Nil	1 (1.52)	Nil	5 (7.58)
III.	Random hospital patients (n = 135)	Nil	113 (83.70)	Nil	Nil	Nil	10 (7.41)	5 (3.70)	7 (5.19)
IV.	Normal healthy subjects (n = 155)	Nil	155 (100)	Nil	Nil	Nil	Nil	Nil	Nil

Pf = *P. falciparum*; Pk = *P. knowlesi*; Pc = *P. cynomolgi*.

Figures in parentheses indicate percentage

*Data from Dutta *et al.* (1982) ; **Data from Srivastava *et al.* (1983).

Table 6. Locality-wise break up of positivity obtained among malaria patients (Group I)

	Locality	E_{492} values Mean ± SD	No. positive at		
			Mean + SD* (E_{492}=0.042)	Mean + 2 SD* (E_{492}=0.054)	Mean + 3 SD* (E_{492}=0.066)
1.	Kalan (n = 55)	0.1200±0.04	53 (96.34)	52 (94.55)	51 (92.73)
2.	Shaktinagar (n = 30)	0.1100±0.02	30 (100)	30 (100)	30 (100)
3.	Ral (n = 41)	0.1150±0.03	41 (100)	41 (100)	41 (100)
4.	Mant (n = 84)	0.1200±0.03	84 (100)	84 (100)	82 (97.62)
5.	Purwa (n = 18)	0.0950±0.03	17 (94.4)	16 (88.89)	16 (88.89)
6.	Chaurmastpur (n = 19)	0.1500±0.045	19 (100)	18 (94.74)	18 (94.74)
7.	Karnal (n = 20)	0.1400±0.04	20 (100)	19 (95)	19 (95)

*Based on values obtained among normal healthy subjects (Group IV)
Figures in parentheses indicate percentage.

all the three antigens; of the remaining 11 cases, 2 gave positive reaction with *P. falciparum* alone, 1 with *P. cynomolgi* alone and 8 with both *P. falciparum* and *P. knowlesi*. Of the 10 cases missed by *P. cynomolgi*, 6 were harbouring *P. falciparum*.

Locality-wise break-up of the positive ELISA results (Table 6) showed that mean E_{492} values differed from one locality to another, although they remained significantly higher in all the localities when compared with the normal values ($p<0.01$).

DISCUSSION

Results of the present investigation clearly indicate that adequately purified *P. cynomolgi* B antigen can be successfully used in the ELISA test for serodiagnosis of human malaria. Antigen prepared from *P. cynomolgi* has been widely used earlier in the indirect fluorescent antibody test (Coudert *et al.*, 1965; Ambroise-Thomas *et al.*, 1976; Agarwal *et al.*, 1983). The present study further corroborates the usefulness of *P. cynomolgi* antigen and extends its use to the ELISA test for seroepidemiology of human malaria. Using this antigen, the ELISA test at 99% confidence limit was found to be highly specific as none of the 155 normal healthy subjects (Group IV) yielded a positive reaction at this cut off point, while a positivity of 96.30% was obtained among the malaria patients (Group I). A very low positivity of 1.43% obtained among patients of pyrexia of varied origin (Group II) and of 11.11% obtained among random hospital patients (Group III) may indicate cases which had a past experience with malaria rather than 'false' positive values.

Our initial results with *P. cynomolgi* B antigen compare favourably with those reported by others using *P. falciparum* antigen (Voller *et al.*, 1980; Mahajan *et al.*, 1981; Spencer *et al.*, 1981; Dutta *et al.*, 1982). The ELISA test with *P. cynomolgi* B antigen should be given a wider trial in areas of *P. vivax* infection to evaluate its field application in other parts of the country. Purified *P. cynomolgi* B antigen as used in this study can be readily prepared in adequate

quantity from the blood of experimentally infected rhesus monkeys and used for large-scale seroepidemiological study.

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REFERENCES

1. Agarwal, S.S., A. Nath, P. Sharma, I.K. Srivastava, S.R. Dwivedi and G.P. Dutta (1983). Comparative evaluation of *Plasmodium knowlesi* and *P. cynomolgi* antigens in the indirect fluorescent antibody test for human malaria. *Indian J. Med. Res.*, 77: 616–622.
2. Agarwal, S.S., A. Nath, P. Sharma, I.K. Srivastava, S.R. Dwivedi, R.L. Yadava and G.P. Dutta (1982). Seroepidemiology of human malaria: Indirect haemagglutination test using *Plasmodium knowlesi* antigen. *Indian J. Malariol.*, 19: 21–25.
3. Agarwal, S.S., P. Sharma, A. Nath, I.K. Srivastava, J. Rai and G.P. Dutta (1981). Indirect haemagglutination and indirect fluorescent antibody tests for human malaria using *Plasmodium knowlesi* antigen. *Indian J. Malariol.*, 18: 67–72.
4. Coudert, J., J.P. Garin, P. Ambroise-Thomas, P. Saliou and M. Minjat (1965). Utilization de *P. cynomolgi* bastianellii dans le serodiagnosticpar immunofluorescence des paludismes humaine. *Bull. Soc. Pathol. Exot.*, 58: 630–639.
5. Dutta, G.P., I.K. Srivastava, P. Sharma, A. Nath, S.S. Agarwal and S.R. Dwivedi (1982). Enzyme linked immunosorbent assay test in the diagnosis of human malaria. *Indian J. Malariol.*, 19: 33–37.
6. Edrissian, G.H., A. Voller and Z. Zoveith (1979). Comparison of enzyme linked immunosorbent assay in the detection of malaria antibody in Southern Iran. *Iranian J. Pub. Health*, 8: 103–110.
7. Francis, V.S., M.M. Gupta and P. Bhat (1982). Evaluation of a crude soluble antigen from *in vitro* culture of *Plasmodium falciparum* for ELISA. *Tropenmed. Parasitol.*, 33: 240–242.
8. Kagan, I.G. (1972). Malaria seroepidemiology and serological diagnosis. *Exp. Parasitol.*, 31: 126–135
9. Lobel, H.O., J.A. Najera, W.I. Chen, P. Munroe and H.M. Mathews (1976). Seroepidemiological investigation of malaria in Guyana. *J. Trop. Med. Hyg.*, 79: 275–284.
10. Mahajan, R.C., N.K. Ganguly, M. Thadani, N. Gilland R.E. Chandanani (1981). Comparison of ELISA with IHA and IFA tests in malaria. *Indian J. Med. Res.*, 73: 874–879.
11. Manawadu, B.R. and A. Voller (1978). Detection and measurement of species specific malarial antibodies by immunofluorescent test. *Trans. R. Soc. Trop. Med. Hyg.*, 72: 463–466
12. Meuwissen, J.H.E.Th. (1974). The indirect haemagglutination test for malaria and its application to epidemiological surveillance. *Bull. WHO*, 50: 277–286.
13. Quakyi, I.A. (1979). Enzyme linked immunosorbent assay and antibodies in malaria. *Trans. R. Soc. Trop. Med. Hyg.*, 73: 105.
14. Spencer, H.C., W.E. Collins, W. Chin and J.E. Skinner (1981). The enzyme linked immunosorbent assay for malaria. III. Antibody response in documented *Plasmodium falciparum* infections. *Am. J. Trop. Med. Hyg.*, 30: 747–750.
15. Srivastava, I.K., P. Sharma, A. Nath, S.S. Agarwal and G.P. Dutta (1983). Enzyme linked immunosorbent assay test with *Plasmodium knowlesi* antigen in diagnosis of malaria. *Indian J. Med. Res.*, 77: 431–436.
16. Voller, A., A. Bartlett and D.E. Bidwell (1976). Enzyme Immunoassays for parasitic diseases. *Trans. R. Soc. Trop. Med. Hyg.*, 70: 98–106.
17. Voller, A., D.E. Bidwell, A. Bartlett and R. Edwards (1977). A comparison of isotopic and enzyme immunoassays for tropical parasitic diseases. *Trans. R. Soc. Trop. Med. Hyg.*, 71: 431–437.
18. Voller, A., R. Cornille-Brogger, J. Storey and L. Molineaux (1980). A longitudinal study of *Plasmodium falciparum* malaria in West African Savanna using the ELISA technique. *Bull. WHO*, 58: 429–438.

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Influence of Progesterone and Estrogen Administration on the Recrudescence Patterns of *Plasmodium knowlesi* Infection in Female Rhesus Monkeys (*Macaca mulatta*) following Initial Subcurative Chloroquine Therapy

G.P. DUTTA[1] and K.K. KAMBOJ[1]

The effect of short-term treatment of female rhesus monkeys (*Macaca mulatta*) with high doses of progesterone or estrogen, on the pattern of recrudescence of *Plasmodium knowlesi*, after initial subcurative chloroquine therapy has been studied. Progesterone (1 mg/kg/day) or estrogen (estradiol dipropionate, 10 µg/kg/day) was administered to groups of nine monkeys by i/m route for 25 days prior to malaria infection and the treatment was continued during the rest of the study. Nine monkeys which served as control, were administered only the vehicle (sterile olive oil, 0.5 ml, i/m) for 25 days. In order to suppress primary infection, chloroquine (9-10 mg base) was administered in 2-4 divided doses between 12-25th day of *P. knowlesi* inoculation, and the patterns of recrudescence were studied.

All the monkeys with progesterone or estrogen showed recrudescence 7-8 days after subcurative therapy with peak parasitaemia levels of 8.50±7.70% and 5.13±3.51% respectively, which were significantly higher than the controls (peak 1.81±0.88%). The total parasitaemia load during recrudescence was 5.10-fold higher in progesterone treated group and 3.46-fold higher in estrogen treated group, as compared to the controls. The duration of major peaks of parasitaemia in both the steroid treated groups was significantly longer. These studies suggest that high doses of steroids tend to slightly delay the development of protective immune responses against *P. knowlesi* in rhesus monkeys.

INTRODUCTION

Oral contraceptives and depot form of progestogens are currently used on a large-scale for contraception. Published reports show that both progesterone and estrogen exhibit a general immunosuppressive action on the host (Krueger, 1972). The PHA-induced blastogenic response of lymphocytes was reported to be depressed in women taking oral contraceptives (Branes *et al.*, 1974; Fitzerald *et al.*, 1973; Hagen and Froland, 1972; Keller *et al.*, 1977), as well as in those receiving estrogen and progesterone substitution therapy for primary ovarian failures (Morishma and Henrich, 1974). Progesterone has been found to cause a depletion in the number of circulating lymphocytes by its direct action on the lymphoreticular tissue (Turcotte *et al.*, 1968). Estrogens have also been reported to suppress CMI and to specifically inhibit cutaneous delayed hypersensitivity reactions (Kappas *et al.*, 1976).

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[1]Central Drug Research Institute, Lucknow-226001, India.

Studies on malaria incidence in Gambian women taking oral contraceptives, suggested that these women maintained a slightly higher level of malaria infection as compared to age matched control group not taking contraceptives (Bray, 1976). However, in this study the blastogenic response of the lymphocytes to PHA was not depressed. Similarly, another study on the immune status of women using oral contraceptives, showed no significant difference in their PHA response of lymphocytes (Ramalakshmi *et al.*, 1979). Although reports in literature are conflicting, the overwhelming evidence tends to suggest, that hormonal steroids might affect the immune status of the host.

Studies on the 'Interactions between oral contraceptives and malaria' carried out in this Institute under the WHO Project--70149B also showed that rhesus monkeys which were administered Norinyl or Ovral-28 for six cycles, when infected with blood induced *P. cynomolgi* B and *P. coatneyi*, tended to maintain higher levels of parasitaemia as compared to the controls not treated with oral contraceptives (Dutta *et al.*, 1984). The present study was planned with a view to provide additional background data on the interaction of hormonal contraceptives and malaria and to understand the effect of individual hormonal steroids (progesterone/estrogen) on the pattern of recrudescences of *P. knowlesi* after initial subcurative therapy with chloroquine.

MATERIAL AND METHODS

Maintenance of rhesus monkeys (Macaca mulatta): Adult female monkeys weighing 5-6 kg, which were negative for tuberculosis as shown by tuberculin test and chest X-ray, were used for this study. They were maintained in air-cooled rooms with 12 hr photoperiodicity (fluorescent lights were kept on from 700-1900 hrs), and were given standard pellet feed supplemented with green vegetables, soaked gram and banana or apple.

Study groups: Normal cycling female monkeys were divided into three groups of nine monkeys each.

1. Control group was administered sterile olive oil (0.5 ml vehicle), daily (i/m).
2. Progesterone group was administered 1 mg/kg progesterone, daily (i/m).
3. Estrogen group was administered 10 μg/kg estradiol dipropionate, daily (i/m).

Steroid treatment was started 25 days before malaria infection and continued till the end of the study.

Malaria infection: Blood induced *Plasmodium knowlesi* (strain W1) infection was used for inoculation of control and steroid treated groups of rhesus monkeys. After 25 days of pretreatment with steroids or vehicle, the animals were inoculated with 50,000 parasitized RBC (ring stage). Course of parasitaemia was recorded daily by examining thin and thick blood smears stained with Giemsa. Parasite counts were made on the basis of 10^4 RBC and expressed as percent parasitaemia.

Recrudescence patterns after subcurative chloroquine therapy: Subcurative doses of chloroquine diphosphate (9.11-10.27 mg chloroquine base in 2-4 divided doses by i/m route) were administered to each monkey during early infection, which resulted in suppression of parasitaemia. Later, all these monkeys showed recrudescence with several peaks of parasitaemia and finally the infection became chronic without further drug intervention (Dutta and Singh, 1980). The schedule of subcurative therapy such as the details of parasitaemia level, chloroquine doses and day of administration are given in Tables 1-3. The peaks of parasitaemia attained during recrudescence, after subcurative therapy in the control and steroid treated groups, were recorded for 50 days of observation period (post-infection) by checking the daily parasitaemia.

Table 1. The pattern of recrudescence of *P. knowlesi* infection in female rhesus monkeys following initial suppression of parasitaemia with subcurative chloroquine therapy (Control group)

S. No.	Subcurative therapy			Recrudescence parasitaemia		
	Parasitaemia %	Chloroquine Dose mg/kg	Chloroquine (On day)	Peak parasitaemia %	Day of peak	Duration (days)
1.	4.2	5.0	(6)	2.1	24	13
	1.8	2.5	(7)	1.2	30	
	2.2	5.0	(13)			
2.	8.1	5.0	(6)	31.2	35	
	5.2	5.0	(8)			
	2.4	2.5	(15)			
3.	1.1	2.5	(6)	1.8	24	18
	1.6	2.5	(7)	2.8	33	
	0.5	2.5	(12)	1.2	40	
	1.2	2.5	(15)			
4.	5.0	5.0	(6)	1.2	23	10
	0.6	2.5	(9)	2.6	27	
	1.2	2.5	(16)			
5.	0.5	2.5	(6)	2.8	30	11
	1.8	5.0	(8)	2.5	32	
	1.6	5.0	(16)			
6.	1.8	2.5	(8)	1.1	30	14
	4.2	2.5	(14)	0.2	38	
	10.2	5.0	(15)			
7.	1.6	2.5	(8)	1.2	37	11
	6.5	5.0	(16)	0.5	41	
	2.1	2.5	(24)			
8.	0.8	2.5	(7)	0.5	35	6
	2.2	2.5	(15)			
	2.4	2.5	(25)			
9.	1.5	2.5	(7)	1.4	29	12
	2.6	2.5	(16)	0.8	37	
	2.5	2.5	(18)			

Vehicle 0.5 ml olive oil (without steroid) was started 25 days prior to infection and was continued throughout the study.
Monkey No. 2 died during recrudescence of parasitaemia.

Table 2. The effect of progesterone administration on the recrudescence of *P. knowlesi* infection in female rhesus monkeys following initial suppression of parasitaemia with subcurative chloroquine therapy (Progesterone group [1 mg/kg/day])

S. No.	Subcurative therapy			Recrudescence parasitaemia		
	Parasitaemia %	Chloroquine Dose mg/kg	(On day)	Peak parasitaemia %	Day of peak	Duration (days)
1.	1.2	2.5	(7)	25.0	30	20
	5.5	5.0	(8)			
	3.5	5.0	(18)			
2.	1.1	2.5	(7)	8.7	32	15
	2.2	2.5	(12)			
	6.5	5.0	(18)			
3.	1.1	2.5	(7)	1.8	30	13
	1.8	5.0	(11)	0.8	37	
	2.7	2.5	(18)			
4.	6.1	5.0	(7)	6.8	23	28
	1.6	2.5	(13)	8.6	32	
	1.2	2.5	(16)	0.8	44	
5.	1.2	5.0	(8)	0.8	31	
	2.8	5.0	(19)	12.1	44	
				50.0	48	
6.	0.8	2.5	(7)	6.8	31	18
	2.8	5.0	(11)	4.1	34	
	2.5	5.0	(19)	3.2	38	
7.	8.2	5.0	(11)	2.6	36	15
	2.1	2.5	(20)	2.8	38	
	3.6	2.5	(25)	4.8	40	
8.	4.2	5.0	(7)	80.0	37	
	10.0	2.5	(8)			
	3.5	2.5	(20)			
9.	8.7	5.0	(11)	3.8	38	14
	2.8	2.5	(24)			

Steroid/vehicle administration was started 25 days prior to infection and was continued throughout the study.
Monkey nos. 5 and 8 died during recrudescence of parasitaemia.

Table 3. The effect of estrogen administration on the recrudescence of *P. knowlesi* infection in female rhesus monkeys following initial suppression of parasitaemia with subcurative chloroquine therapy (Estrogen group [estradiol dipropionate 10 μg/kg /day])

S. No.	Subcurative therapy			Recrudescence parasitaemia		
	Parasitaemia	Chloroquine		Peak parasitaemia	Day of peak	Duration
	%	Dose mg/kg	(On day)	%		(days)
1.	1.2	2.5	(6)	1.9	30	18
	5.1	5.0	(7)	1.8	34	
	1.4	2.5	(14)	1.2	37	
	4.2	5.0	(17)			
2.	1.1	2.5	(6)	0.8	24	22
	2.2	5.0	(12)	10.4	24	
				2.6	30	
				0.8	38	
3.	1.2	2.5	(7)	1.2	22	
	2.8	5.0	(12)	0.5	28	
				4.1	46	
				45.1	49	
4	1.1	5.0	(7)	0.4	26	
	1.4	2.5	(13)	22.1	31	
	2.6	5.0	(18)	48.3	36	
5.	0.8	2.5	(7)	0.2	23	27
	2.8	5.0	(11)	7.6	30	
				0.4	39	
				1.6	47	
6.	3.1	2.5	(7)	5.2	37	16
	2.8	2.5	(13)	1.1	42	
	1.5	2.5	(25)			
7.	2.8	2.5	(6)	1.5	32	12
	0.8	2.5	(7)	7.2	37	
	2.1	2.5	(19)	7.5	40	
	2.8	2.5	(24)			
8.	2.2	2.5	(7)	1.5	30	18
	6.2	2.5	(10)	0.8	38	
	4.5	2.5	(20)			
9.	0.8	2.5	(7)	1.8	27	22
	4.8	2.5	(13)	1.6	35	
	2.8	2.5	(19)	1.2	38	

Steroid/vehicle administration was started 25 days prior to infection and was continued throughout the study.
Monkey nos. 3& 4 died during recrudescence of parasitaemia.

RESULTS

The details of the data on recrudescence in individual monkeys after subcurative chloroquine therapy, and the duration of major peaks as judged by the period during which the parasitaemia remained above 0.1% level in control, progesterone and estrogen treated monkeys, are given in Figs. 1-3, and Tables 1-3 and the statistical significance of the data in the three groups is presented in Table 4.

In the control group, recrudescence was observed 5-10 days (average 7.88 days) after the last subcurative dose of chloroquine, whereas in the progesterone group the parasitaemia appeared between 4-12 days (average 8.33 days) and in the estrogen group between 6-10 days (average 7.77 days) after the last dose.

Further, the peak parasitaemia during recrudescence in the control monkeys was 1.81 ± 0.88% (range 0.2 - 2.8%), and the duration of major peaks was 11.87 ± 3.44 days (range 6-18 days). In the progesterone group, the peaks of parasitaemia showed an average of 8.50 ± 7.70% (range 0.8 - 25.0%), and the average duration of peaks was 17.8 days (range 13-28 days). Similarly in the estrogen group, the peaks of parasitaemia showed an average of 5.13 ± 3.51% (range 0.2 - 10.4%), and the average duration of peaks was 19.1 days (range 12-28 days). The peak levels of parasitaemia in 5 monkeys (one control, two progesterone treated and two estrogen treated) which shot up to high levels during recrudescence, resulted in the death of the monkeys and they were not taken into account for comparing the peak parasitaemia levels.

The total cumulative parasitaemia load during recrudescence (upto 50 days of infection) was 3.46 to 5.10-fold higher in both the steroid treated groups, as compared to controls (Table 5).

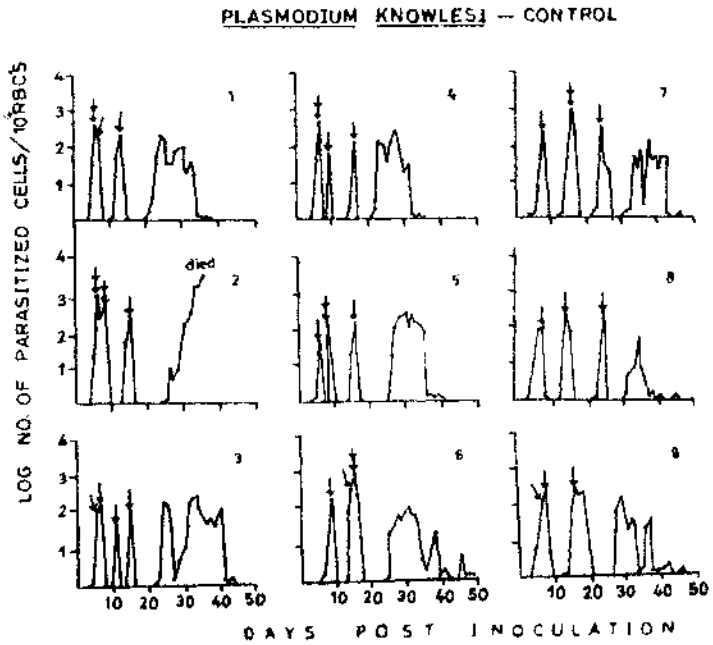

Fig. 1: Control group: Course of *P. knowlesi* infection in 9 monkeys undergoing treatment with 0.05 ml sterile olive oil (vehicle)↓= 2.5 mg chloroquine↓= 5.0 mg chloroquine).

RESULTS

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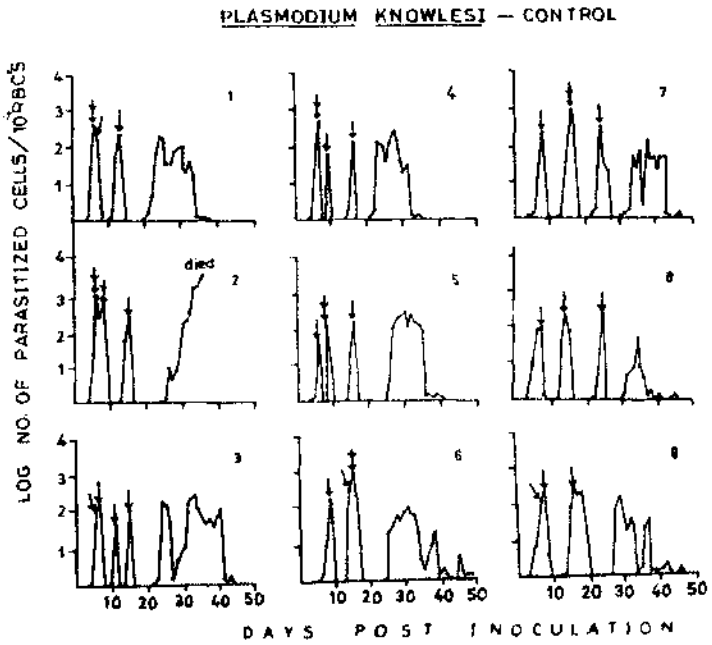

Fig. 1- Control group: Course of *P. knowlesi* infection in 9 monkeys undergoing treatment with 0.05 ml sterile olive oil (vehicle)↓= 2.5 mg chloroquine)↓= 5.0 mg chloroquine).

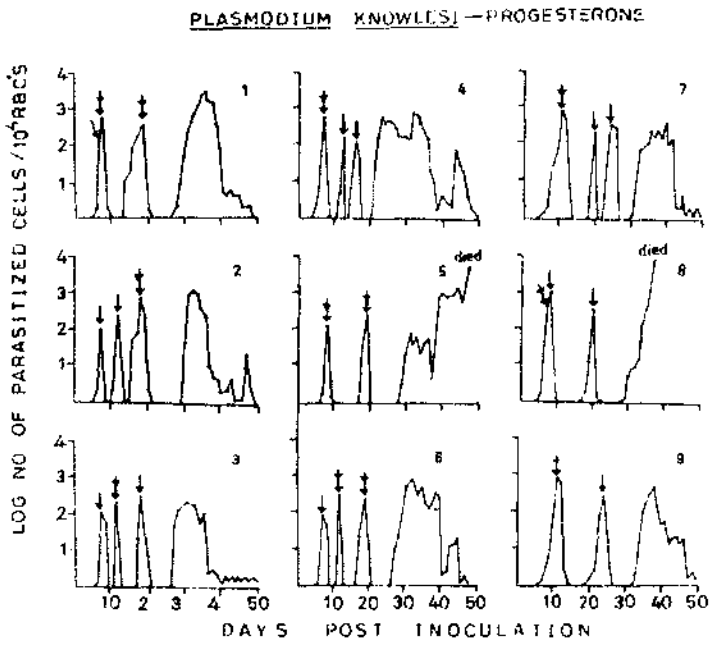

Fig. 2: Progesterone group: Course of *P. knowlesi* infection in 9 monkeys undergoing treatment with 1 mg/kg progesterone (↓= 2.5 mg chloroquine, ↓↓= 5.0 mg chloroquine).

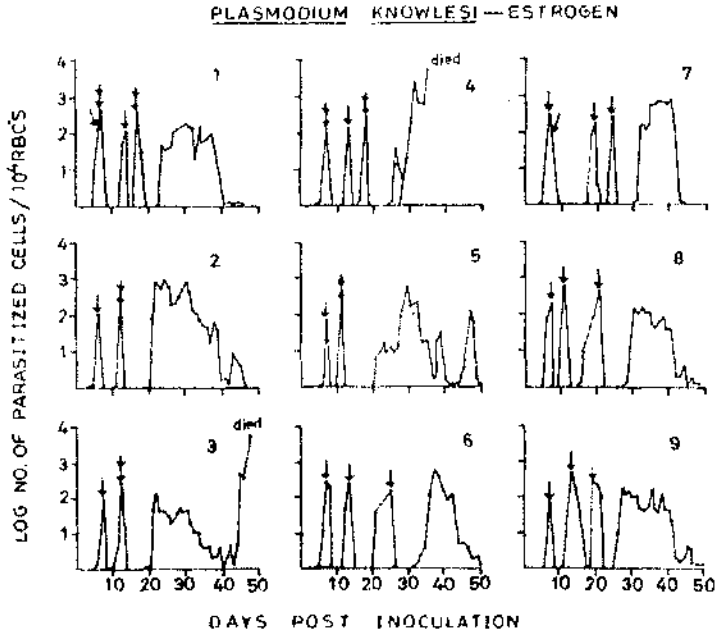

Fig. 3: Estrogen group. Course of *P. knowlesi* infection in 9 monkeys undergoing treatment with 10 μg/kg estradiol dipropionate (↓= 2.5 mg chloroquine, ↓↓= 5.0 mg chloroquine).

Table 4. Statistical analysis of the data on the effect of progesterone and estrogen administration on the recrudescences of *P. knowlesi* infection in female rhesus monkeys following initial suppression of parasitaemia with subcurative chloroquine therapy (Mean ± SD)

Group	No.	Subcurative Chloroquine therapy		Recrudescence parasitaemia					
		Total dose of drug mg/kg	Last day of drug	Day after infection	Day after last dose of drug	Major peak %	Day of major peak	Duration of recrudescence*	Mortality after recrudescence
1. Control	9	10.27±1.95	17.44±4.21	25.33±4.09	7.88±1.53	1.81±0.88	31.11±4.22	11.87±3.44	1/9
2. Progesterone	9	10.27±1.50	19.66±2.95	27.88±3.95	8.33±2.50	8.50±7.70	36.65±5.57	17.57±5.19	2/9
		NS	NS	NS	NS	P <0.05	NS	P<0.05	
3. Estrogen	9	9.11±2.83	17.55±5.12	25.33±4.15	7.77±1.56	5.13±3.51	33.66±7.66	19.28±4.05	2/9
		NS	NS	NS	NS	P<0.001	NS	P<0.02	

*Monkeys which died due to high parasitaemia during recrudescence were not considered for statistical analysis of the data on recrudescence.

Table 5. Cumulative parasite load during recrudescence of parasitaemia (*P. knowlesi*) in rhesus monkeys treated with progesterone/estrogen and controls following subcurative therapy

Treatment group	No. of monkeys*	Period of observation	Cumulative parasitaemia load/10^4RBC
Control	8	50	742.60
Progesterone	7	50	3786.10
Estrogen	7	50	2559.00

*Survived recrudescence

Control vs Progesterone	5.10	Fold increase
Control vs Estrogen	3.46	
Estrogen vs Progesterone	1.47	

DISCUSSION

P. knowlesi infection was used in this study to monitor the effect of progesterone and estrogen individually, on the development of protective immune response; since it can produce both fatal infection in untreated monkeys, and a chronic infection after appropriate initial subcurative therapy with chloroquine (Dutta and Singh, 1980). It has been suggested that subcurative therapy of *P. knowlesi* infection stimulates host immune responses which modify the course of *P. knowlesi* infection, resulting in chronicity. After cessation of chloroquine therapy, a few waves of parasitaemia are observed during recrudescence, but the successive peaks are suppressed by the increasing protective immune responses of the host. The results of the present study clearly show that the major peak levels of parasitaemia attained during recrudescence were 5.10-fold higher in the progesterone treated group and 3.46-fold higher in the estrogen group as compared to the controls, and the difference between peak heights in steroid treated groups and the controls was statistically significant. Further, the duration of major peaks was significantly longer in steroid treated groups as compared to controls. The present study suggests that administration of high doses of progesterone or estrogen could delay the development of protective immune response in malaria, resulting in a transient but significantly higher parasitaemia load during recrudescence. However, the infection is finally controlled effectively in both the steroid treated groups and the controls, resulting in chronicity of infection.

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REFERENCES

1. Barnes, E.W., A.C. MacGuish, M.B. Loudon, J. Jordon and W.J. Irvine (1974). Phyto-haemagglutinin-induced lymphocyte transformation and circulating autoantibodies in women taking oral contraceptives. *Lancet*, 1: 898.
2. Bray, R.S. (1976). Some immune responses of Gambian women taking the combined oral contraceptive pill. *Contraception*, 13: 417.
3. Dutta, G.P. and P.P. Singh (1980). Immune status of rhesus monkeys infected with *Plasmodium knowlesi* after curative and suppressive chloroquine therapy. *Indian J. Med. Res.*, 72: 23.
4. Dutta, G.P., S.K. Puri, K.K. Kamboj, S.K. Srivastava and V.P. Kamboj (1984). Interaction between oral contraceptives and malaria. *Bull. WHO*, 62: 931-939.
5. Fitzerald, P.H., A.F. Pickering and D.N. Fergusion (1973). Depressed lymphocyte response to PHA in long term users of oral contraceptives. *Lancet*, 1: 615.
6. Hagen, C. and A. Froland (1972). Depressed lymphocyte response to PHA in women taking oral contraceptives. *Lancet*, 1: 1185.
7. Kappas, A., H.E.H. Jones and I.M. Roitt (1963). Effect of steroid sex hormones on immunological phenomena. *Nature*, 198: 902.
8. Keller, A.J., W.J. Irvine, J. Jorden and N.B. Loudon (1977). Phyto-haemagglutinin-induced lymphocyte transformation in oral contraceptive users. *Obstet. Gynaecol.*, 49: 83.
9. Krueger, G.R.F. (1972). Morphology of chemical immunosuppression. *Adv. Pharmacol. Chemother.*, 10: 1.
10. Morishma, A. and R.T. Henrich (1974). Lymphocyte transformation and oral contraceptives. *Lancet*, 2: 646.

11. Ramalakshmi, B.A., V. Jagadeesan and K. Prema (1979). Immune studies in oral contraceptive users. *Contraception*, 20: 417.

12. Turcotte, J.G., R.F. Haines, G.L. Brody, T.J. Meyer and S.A. Schwartz (1976). Immunosuppression with medroxy-progesterone acetate. *Transplantation*, 6: 248.

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Biochemical Changes in Cellular Constituents and some Enzymes in Host-tissues from *Plasmodium knowlesi* Infected Rhesus Monkey (*Macaca mulatta*)

RAHUL SRIVASTAVA[1], NALINI SAXENA[1], V.C. PANDEY[2] and G.P. DUTTA[1]

Changes in the cellular constituents as well as some of the marker enzymes of carbohydrate metabolism, proteinases, phosphatases, nucleases, GOT and GPT in liver, kidney and spleen of rhesus monkey following acute *Plasmodium knowlesi* infection have been studied. As a result of malaria infection, there was significant depletion of total carbohydrate content in the liver and kidney. Spleen showed elevated glucose levels. The levels of glycolytic and TCA cycle enzymes were generally lowered in all the malaria infected tissues and pentose phosphate cycle key enzyme (G-6-PD) was stimulated in the liver but there was marked decrease in the spleen. There was also a depletion of total protein and phosphorous contents in liver and kidney during malaria infection with a concomitant increase of proteinases and phosphatases. The spleen which is the main target of immunopathology in malaria, showed net increase in protein and phosphorous contents and correspondingly no activation of proteinases or phosphatases were observed. The activities of both the acid and alkaline DNases in all the infected tissues were elevated and consequently there was a decline of total DNA content of tissues following malaria infection. Total RNA content showed major decline in spleen and less so in kidney and liver and the corresponding RNA hydrolysing enzyme showed marked increase in kidney in comparison to spleen. Lipid analysis showed an increase in the level of total lipids, triglycerides and phospholipids in the liver, and that of cholesterol and phospholipids in infected spleen.

INTRODUCTION

A limited amount of information is available in literature regarding changes in biochemical parameters of the host during primate malaria. Changes in only a few cellular constituents of different tissues during malaria infection have been studied viz., lipid profile of the liver of mice infected with *P. berghei* (von Brand and Mercado, 1958; Rao *et al.*, 1967) and liver of rhesus monkey infected with *P. knowlesi* (Angus *et al.*, 1971). Alteration in nucleic acid contents of liver, kidney and spleen of rat infected with *P. berghei* (Buangener, 1965) and liver of chick infected with *P. gallinaceum* (Rama Rao, 1978) were also studied.

Mercado and von Brand (1954) demonstrated lowering of glycogen synthesis in rat due to *P. berghei* infection. Similar findings were reported for liver of rhesus monkey due to *P. knowlesi* infection (Maegraith, 1968; Devakul and Maegraith, 1958) and in albino rat infected with *P. berghei* (Chatterji and Sen Gupta, 1957).

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Division of Biochemistry[2] and Microbiology[1]
Central Drug Research Institute, Lucknow-226001, India.

Malaria infection is also known to cause hypoglycaemia in the host particularly during acute state of infection (Chatterji and Sen Gupta, 1957; Devakul, 1960).

Changes in protein contents of liver and spleen in *Mastomys natalensis* (Saxena *et al.*, 1981) and mice (Sharma *et al.*, 1979) during *P. berghei* infection, have also been reported. Rao *et al.* (1967) noticed the changes in phospholipid contents of mouse spleen due to *P. berghei* infection. Impairment of respiratory function and oxidative phosphorylation of the liver mitochondria, of mice and rats, during malaria infection has also been reported by some workers (Basu *et al.*, 1962; Riley and Deegan, 1960; Riley and Maegraith, 1962; Maegraith, 1954).

In view of the fragmentary information in literature about different biochemical parameters of malaria affected tissues, an attempt has been made in this study to analyse some of the biochemical changes in liver, spleen and kidney of rhesus monkey infected with *P. knowlesi*.

MATERIAL AND METHODS

Experimental animal: Adult rhesus monkeys (*Macaca mulatta*) of either sex, weighing about 4–6 kg were kept under 12 hrs photoperiodicity with fluorescent lights on from 7.00 hrs to 19.00 hrs. Rhesus monkeys used in the study were certified to be free from tuberculosis as shown by negative tuberculin test and chest X-ray. The monkeys were maintained on normal diet consisting of pellets, fruit, green vegetables and soaked gram.

Malaria infection: *Plasmodium knowlesi* (W_1) strain kindly donated by Prof. P.C.C. Garnham, FRS was used for infecting the monkeys. They were inoculated with 1×10^5 parasitized red blood cells from infected donor monkey and the course of parasitaemia was recorded from thin smears stained with Giemsa stain. Parasitaemia was expressed as parasitized cells per 100 RBCs.

The animals were sacrificed at acute parasitaemia (40–60% schizont infection) by cardiac puncture and the tissues viz., liver, kidney and spleen were collected in ice and processed immediately for various biochemical assays. Normal animals were also sacrificed along with the infected animals in each set of experiments.

Preparation of homogenate: 10% (w/v) homogenate was made by homogenizing the fresh tissues in Potter Elvehjem homogenizer at 4°C in pre-chilled distilled water. The homogenate was centrifuged at 4,000 g for ten minutes in cold and supernatant was separated for enzyme assays to serve as source of enzyme.

Estimation of cellular constituents: Cellular constituents were estimated by the methods given by following workers:

1. Total carbohydrate estimation (Montgomery, 1957)
2. Glycogen isolation (Good *et al.*, 1933)
3. Glycogen estimation (Montgomery, 1957)
4. Glucose estimation (Bergmeyer and Benut, 1963)
5. Protein estimation (Lowry *et al.*, 1951)
6. Phosphorus estimation (Bartlett, 1959)
7. Nucleic acids isolation and estimation (Schneider, 1945)
8. Lipids extraction (Folch *et al.*, 1957)
9. Cholesterol estimation (Wybenga *et al.*, 1970)
10. Triglyceride estimation (Gottfried and Rosenberg, 1973)
11. Phospholipid estimation (Bartlett, 1959; Merinetti, 1962).

Estimation of enzymes: Enzymes were estimated by the methods given by following workers:

1. Glycogen phosphorylase, E.C. No. 2.4.1.1. (Sutherland and Wosilait, 1956)
2. Hexokinase, E.C. No. 2.7.1.1. (Crane and Sols, 1953)
3. Aldolase, E.C. No. 4.1.2.13. (Sibley and Lehninger, 1949)

4. Lactate dehydrogenase (LDH), E.C. No. 1.1.1.27. (Wroblewski and LaDue, 1955)
5. Malate dehydrogenase (MDH), E.C. No. 1.1.1.37. (Ochoa, 1955)
6. Succinic dehydrogenase (SDH), E.C.No. 1.3.99.1. (Slater and Bonner, 1952)
7. Glucose-6-phosphate dehydrogenase (G-6-PDH), E.C.No. 1.1.1.49. (Demoss, 1955)
8. Proteinase, (Anson, 1938)
9. Acid phosphatase, E.C.No. 3.1.3.2., alkaline phosphatase, E.C.No. 3.1.3.1. (Bessey *et al.*, 1946; Andersch and Szczypinski, 1947)
10. Ribonuclease, E.C.No. 2.7.7.16. (Ishihara *et al.*, 1967)
11. Deoxyribonuclease, E.C.No. 3.1.4.5. (Irie *et al.*, 1966)
12. Glutamate oxaloacetate transaminase, E.C. No. 2.6.1.1. and glutamate pyruvate transaminase, E.C.No. 2.6.1.2. (Wotton, 1964).

Enzyme activity and specific activity: One unit of enzyme activity is defined as u moles of product formed per min. excepting proteinase where the unit is defined as μ moles of product formed per 30 mins. by the enzyme under the experimental conditions. Specific activity is expressed in units of enzyme activity per mg protein.

RESULTS

Major biochemical changes in liver, kidney and spleen of rhesus monkey following acute *P. knowlesi* infection have been investigated and the data on the level of biochemical constituents and enzymes in normal and infected tissues expressed as percentage change in Tables 1 and 2. The carbohydrate metabolism of the monkey was severely disturbed as a result of malaria infection. This was evidenced by a highly significant depletion of total carbohydrates (-86.87%), glycogen reserve (-96.76%) and glucose level (-89.97%) in the liver. The kidney also showed a significant depletion of these three constituents to the extent of -24.15, -29.56 and -66.69%, respectively. In the spleen, the changes in total carbohydrates (+10.00%) and glycogen (-4.62%) were not well marked but a highly significant increase in glucose level (+575.34%) was observed. The enzyme glycogen phosphorylase which regulates the glycogen mobilization, was significantly elevated in the liver (+24.39%) and kidney (+50.00%) and its level was depressed in spleen (-58.83%).

There was a slight decrease in the total protein content of liver (-6.74%) and kidney (-4.26%), contrarily, a highly significant increase of protein content in the spleen (+47.96%) was observed due to malaria infection.

Nucleic acid (total DNA and RNA) content exhibited a uniform decrease in all the three tissues during malaria infection. The decrease in total DNA in liver (-40.76%), kidney (-35.66%) and spleen (-44.58%) was much more pronounced than that of total RNA which was to the extent of -3.52% in liver, -27.14% in kidney and 27.70% in the spleen.

Total phosphorous content showed decrease in case of liver (-16.07%) and kidney (-32.41%) while there was a steep rise in the phosphorous content of spleen (+43.02%).

Assay for total lipids showed an increase in both liver (+31.04%) and kidney (+18.64%), while spleen showed a decline (-16.61%) after malaria infection. Cholesterol level showed a major increase in spleen (+57.43%). Triglyceride levels were also elevated in liver (+40.01%) and kidney (+15.74%) but decreased in spleen (-19.16%). Phospholipids also showed a major increase in the spleen (+42.33%), whereas changes in liver (+11.15%) and kidney (-7.33%) were marginal.

All the three enzymes of glycolytic cycle namely - hexokinase, aldolase and lactic dehydrogenase showed a marked decrease in all the tissues. The decrease in hexokinase was -68.35, -49.39 and -30.55%, aldolase -59.49, -32.99 and -28.87% and that of LDH was -27.77, -32.99 and

Table 1. Levels of enzymes of Liver, Kidney and Spleen of normal and *P. knowlesi* infected monkeys

S. No.	Enzyme	LIVER			KIDNEY			SPLEEN		
		Normal	Infected	% change/ p–value	Normal	Infected	% change/ p–value	Normal	Infected	% change/ p–value
1.	Glycogen phosphorylase	$5.70\times10^{-3}\pm0.70\times10^{-5}$	$7.09\times10^{-3}\pm0.09\times10^{-4}$	+24.39 p<0.01	$5.72\times10^{-3}\pm0.16\times10^{-3}$	$8.58\times10^{-3}\pm0.80\times10^{-3}$	+50.00 p<0.01	$21.45\times10^{-3}\pm0.48\times10^{-4}$	$5.83\times10^{-3}\pm0.20\times10^{-4}$	-58.83 p<0.01
2.	Hexokinase	$86.34\times10^{-3}\pm16.47\times10^{-3}$	$27.33\times10^{-3}\pm5.41\times10^{-3}$	-68.35 p<0.01	$69.92\times10^{-3}\pm7.69\times10^{-3}$	$35.39\times10^{-3}\pm5.01\times10^{-3}$	-49.39 p<0.01	$100.69\times10^{-3}\pm3.65\times10^{-3}$	$69.92\times10^{-3}\pm7.69\times10^{-3}$	-30.55 p<0.01
3.	Aldolase	1.98±0.15	0.80±0.16	-59.49 p<0.01	1.14±0.14	0.82±0.15	-32.99 p<0.01	0.47±0.06	0.34±0.06	-28.87 p<0.01
4.	Lactate dehydrogenase	0.51±0.02	0.37±0.08	-27.77 p<0.01	1.35±0.04	0.90±0.08	-32.99 p<0.01	0.75±0.04	0.48±0.07	-36.41 p<0.01
5.	Malate dehydrogenase	2.81±0.28	1.52±0.05	-45.74 p<0.01	2.34±0.05	2.190±0.23	-6.24 p<0.01	0.66±0.06	0.47±0.03	-28.51 p<0.01
6.	Succinate dehydrogenase	12.29±1.09	7.09±1.37	-42.31 p<0.01	19.99±0.29	16.29±1.97	-18.51 p<0.01	10.44±0.40	6.84±0.87	-34.47 p<0.01
7.	Glucose 6PO4 dehydrogenase	$0.64\times10^{-3}\pm0.11\times10^{-3}$	$1.40\times10^{-3}\pm0.33\times10^{-3}$	+118.75 p<0.01	$1.23\times10^{-3}\pm0.35\times10^{-3}$	$1.19\times10^{-3}\pm0.21\times10^{-3}$	+5.59 p>0.5	$9.40\times10^{-3}\pm0.87\times10^{-3}$	$3.76\times10^{-3}\pm0.96\times10^{-3}$	-60.00 p<0.01
8.	Acid proteinase	0.09±0.01	0.10±0.01	+11.1 p<0.1	0.11±0.01	0.11±0.01	Nil N.S.	0.18±0.03	0.14±0.04	-20.44 p>0.5
9.	Alkaline proteinase	0.04±0.01	0.09±0.02	+125.0 p<0.01	0.05±0.01	0.08±0.02	+60.0 p<0.01	0.08±0.01	0.05±0.01	-36.84 p<0.02
10.	Acid phosphatase	0.12±0.01	0.14±0.02	+16.6 p<0.02	0.10±0.01	0.15±0.01	+50.00 p<0.05	0.52±0.09	0.18±0.01	-66.15 p<0.01
11.	Alkaline phosphatase	0.04±0.02	0.15±0.03	+273.17 p<0.01	0.07±0.01	0.11±0.03	+57.0 p<0.01	0.20±0.03	0.08±0.02	-59.90 p<0.01
12	Acid RNase	81.44±11.74	156.66 ±18.17	+92.36 p<0.01	186.31±9.88	204.9±14.03	+9.9 p<0.05	295.85±12.08	240.50 ±19.09	-18.71 p<0.01
13.	Alkaline RNase	16.57±6.71	59.80±10.55	+260.89 p<0.01	14.37±3.22	93.58±7.35	+551.22 p<0.01	73.07±6.39	61.08±6.37	-16.41 p<0.01
14.	Acid DNase	18.95±2.77	35.22±5.29	+85.86 p<0.01	33.26±2.62	35.06±7.62	+2.40 p>0.5	26.42±2.41	51.32±12.50	+94.25 p<0.01
15.	Alkaline DNase	12.61±1.43	83.14±4.69	+559.32 p<0.01	46.63±4.08	80.73±2.13	+73.13 p<0.01	15.94±4.02	59.71±7.18	+274.59 p<0.01
16.	GOT	3.48±0.18	3.22±0.49	-7.47 p<0.5	4.97±0.30	4.45±0.22	-10.46 p<0.02	5.13±0.16	4.66±0.60	-9.16 p<0.2
17.	GPT	22.61±1.46	19.95±1.99	-11.76 p<0.2	25.27±1.06	23.80±2.13	-5.82 p<0.5	11.97±1.73	12.50±5.98	+4.43 p>0.5

(1) Results are expressed as Unit/mg protein.

(2) Results are mean values ± SD of six sets of animals analysed on the different days of infection along with control. (Each set contained one healthy and one infected animal).

(3) N.S. — Not Significant.

Table 2. Levels of cellular constituents of liver, kidney and spleen of normal and *P. knowlesi* infected monkeys

S. No	Cellular constituents	LIVER			KIDNEY			SPLEEN		
		Normal	Infected	% change/ p-value	Normal	Infected	% change/ p-value	Normal	Infected	% change/ p-value
1.	Total Carbohydrate	66.33±1.76	8.71±0.80	-86.87 p<0.01	7.50±1.08	5.69±0.54	- 24.15 p<0.01	8.00±0.52	8.80±0.40	+10.00 p<0.05
2.	Total Glycogen	40.90±2.78	1.37±0.17	-96.66 p<0.01	1.10±0.14	0.78±0.10	-29.56 p<0.01	1.32±0.19	1.26±0.36	-4.62 p<0.05
3.	Glucose	21.99±1.10	2.21±0.11	-89.97 p<0.01	1.32±0.21	0.44±0.02	-66.69 p<0.01	0.59±0.00	3.97±0.41	+575.34 p<0.01
4.	Total Protein	215.00±5.13	200.15 ±15.18	-6.74 p<0.1	145.00 ±0.00	138.83 ±14.19	-4.26 p<0.50	122.00 ±1.15	180.50 ±8.41	+47.96 p<0.01
5.	Total Phosphorous	2.79±0.63	2.35±0.27	-16.07 p<0.2	2.09±0.18	1.41±0.24	-32.41 p<0.05	2.39±0.15	3.42±0.17	+43.02 p<0.01
6.	Total RNA	19.05±2.57	18.38±2.11	-3.52 p>0.5	11.87±1.63	8.65±2.89	-27.14 p<0.1	23.44±2.77	16.95±3.33	-27.70 p<0.02
7.	Total DNA	3.38±0.49	2.00±0.52	-40.76 p<0.01	3.39±0.85	2.18±0.33	-35.66 p<0.01	7.23±1.76	4.01±0.67	-44.58 p<0.01
8.	Total Lipid	50.10±0.71	65.65±5.44	+31.04 p<0.01	41.30±1.27	49.00 ±7.72	+18.64 p<0.05	56.90±3.81	47.45±5.72	-16.61 p<0.02
9.	Cholesterol*	53.23±1.14	51.51±1.81	-3.23 p<0.20	46.55±2.44	43.92 ±4.30	-5.65 p<0.05	50.08±4.06	78.84±2.39	+57.43 p<0.01
10.	Triglyceride*	70.23±3.49	98.33±7.03	+40.01 p<0.01	40.15±3.47	46.47 ±2.90	+15.74 p<0.02	46.50±3.99	37.59±2.03	-19.16 p<0.01
11.	Phospholipid	17.31±2.01	19.24±1.03	+11.15 p<0.1	15.29±2.48	14.17 ±3.49	-7.33 p<0.5	14.93±1.03	21.25±2.45	+42.33 p<0.01

(1) Results are mean values ± SD of six sets of infected and normal monkeys analysed on different days. (Each set contained one normal and one infected animal).

(2) Results are expressed as mg/g of fresh tissue.

(3) *Values expressed as mg/100 ml of homogenate (10% w/v).

-36.41% in malaria affected liver, kidney and spleen, respectively.

The TCA cycle enzymes, namely MDH and SDH also showed a general decline during malaria infection, the level of MDH being -45.74, -6.24 and -28.51% and that of SDH -42.31, -18.51 and -34.47% in liver, kidney and spleen, respectively.

Glucose-6-phosphate dehydrogenase, a key enzyme of pentose phosphate pathway, showed an increase of +118.7 and +5.59% in liver and kidney respectively, and a decline of -60.00% in the spleen.

As regards the alterations in specific activities of hydrolases, the data obtained indicated a noticeable increase in the acid and alkaline proteinases and phosphatases in liver and kidney due to *P. knowlesi* infection. It was interesting to note that the increase in alkaline phosphatase of the infected liver was exceptionally high. On the contrary, there was a marked decrease in both proteinase and phosphatase activities in the infected spleen. The activity of both acid and alkaline RNases was elevated in the infected liver and kidney, but the increase in alkaline RNase in kidney was highly significant. In case, of spleen, the level of both the RNases was decreased. The increased levels of DNase (acid and alkaline) was recorded in all the tissues, alkaline DNase showed highly significant increase in both liver and spleen.

The changes in the level of GOT and GPT in the infected tissues were marginal. Both the enzymes showed a slight decrease in all the tissues, excepting spleen which showed a marginal rise in GPT level.

DISCUSSION

Present findings establish that during malaria infection, significant biochemical disorders occur in the metabolism of carbohydrate, lipids, nucleic acids and phosphorous in the three tissues namely – liver, kidney and spleen. Liver, which is an important site for storage of reserve carbohydrate, is severely depleted of its glycogen content due to acute *P. knowlesi* infection in rhesus monkeys. There was also a fall in the glycogen level of the kidney. Depletion of liver carbohydrate reserves of the host during malaria infection in rodents had been reported by several investigators (Mercado and von Brand, 1954; Chatterji and Sen Gupta, 1957; Devakul and Maegraith, 1958; Saxena *et al.*, 1981).

Decrease in the level of glucose in liver and kidney during *P. knowlesi* infection in *M. mulatta* suggested a fast mobilization of the carbohydrate moiety resulting in hypoglycemic condition in later stages of infection. Although the carbohydrate and glycogen levels of spleen were the same, the glucose level was significantly increased due to the inability of the tissue to metabolise glucose.

The present findings clearly demonstrate that the depletion of liver and kidney glycogen is primarily due to the excessive breakdown of glycogen by the polysaccharide degrading enzyme, glycogen phosphorylase. Saxena *et al.* (1981) also demonstrated an increase in the level of glycogen phosphorylase of *P. berghei* infected liver of *Mastomys natalensis*. The spleen is not an important site for the storage of glycogen, however, the decrease in its glycogen phosphorylase activity and increase in glucose content is difficult to explain at present. The increased glucose level in the spleen may be due to the accumulation of parasitized cells and other cell debris.

The marker enzymes of glycolysis and TCA cycle were markedly decreased due to *P. knowlesi* infection in monkey tissues suggesting that both glycolysis and TCA cycle of rhesus monkey liver, kidney and spleen were partially affected due to acute malaria infection. Basu *et al.* (1962) reported that malate, glutamate and isocitrate dehydrogenase levels of rat liver during *P. berghei* infection were not altered significantly.

On the contrary, a depression in the respiratory enzymes of the liver of mice infected with *P. berghei* was noted by Aviado *et al.* (1969). There are reports about the involvement of liver mitochondria during malarial infection in rats (Maegraith, 1954; Basu-Mallik *et al.*, 1962) and mice (Riley and Deegan, 1960 ; Riley and Maegraith, 1962) indirectly suggesting the involvement of the biocatalyst. Maegraith *et al.* (1962), however, observed lowering of SDH with no change in cytochrome oxidase activity in liver of monkey infected with *P. knowlesi*.

It was observed that the remaining alternative pathway of direct oxidation of glucose i.e., pentose phosphate pathway, had also been affected due to *P. knowlesi* infection. The observation of increased G-6-PDH activity is an index of enhanced pentose phosphate pathway activity in the liver and kidney suggesting that under malaria infection, the liver becomes more dependent on direct oxidation of glucose. In malaria infected RBCs, increased pentose phosphate pathway activity had been reported by Fletcher and Maegraith (1962), Herman *et al.* (1966) and Srivastava *et al.* (1982). The pentose phosphate pathway of the spleen alongwith its glycolysis and TCA cycle enzymes was inhibited due to malaria infection.

The present study also demonstrated that *P. knowlesi* infection in monkeys had significantly altered the levels of protein, phosphorous and nucleic acids of the host. These constituents were considerably decreased in infected liver and kidney. The spleen was an exception in displaying a net increase in total protein content due to acute infection, which may be attributed either to the synthesis of fresh protein or to the accumulation of foreign bodies (i.e., macrophages and parasites etc.). Sharma *et al.* (1979) also reported an increase in the protein content of spleen, and decrease in liver protein due to *P. berghei* infection in mice and *Mastomys natalensis*.

The stress of malaria infection leading to the alterations in macromolecules directly indicated the involvement of respective hydrolases in the metabolism of these macromolecules. Our data further supports the involvement of alkaline hydrolases in metabolism of macromolecules in preference to acid hydrolases during malaria. Buangener (1965) had also reported a decrease in the nucleic acids and nucleases of liver, kidney and spleen due to *P. berghei* infection in rat. However, Rama Rao (1978) reported an increase in nucleic acid during malaria infection in chicks.

Chatterji and Sen Gupta (1957) studied the behaviour of alkaline phosphatase in rat liver infected with *P. berghei* and found no appreciable changes in host due to infection.

With the rise in parasitaemia various tissues of the host are damaged to varying degrees. In the present study lipid content of infected spleen was found to be depleted which may be due to the increased susceptibility of these tissues to oxidative damage under the stress of malaria infections (Chauhan *et al.*, 1981). Von Brand and Mercado (1958) and Rao *et al.* (1969) also reported an increase in lipid content of *P. berghei* infected rat liver and attributed this to the accumulation of triglycerides in the liver. On the contrary, liver lipids were found to be decreased due to *P. berghei* infection by many workers (Sharma *et al.*, 1979; Saxena *et al.*, 1981). Our findings are in agreement with the findings of von Brand and Mercado (1958) and Rao *et al.* (1969) who also reported an increase in liver and kidney lipids.

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REFERENCES

1. Andersch, M.A. and A.J. Szczypinski (1947). Use of p-nitrophenyl phosphate as the substrate in determination of serum acid phosphatase. *Am. J. Clin. Pathol.*, **17**: 571
2. Angus, M.G.N., K.A. Fletcher and B.G. Maegraith (1971). Studies on lipids of *Plasmodium knowlesi* infected rhesus monkeys (*Macaca mulatta*) IV: Changes in erythrocyte lipids. *Ann. Trop. Med. Parasitol.*, **65**: 429.
3. Anson, M.L. (1938). The estimation of pepsin, papain and cathepsin with hemoglobin. *J. Gen. Physiol.*, **22**: 79.
4. Aviado, D.M., Y.W. Cho and J.M. Smith (1969). Pathological physiology and chemotherapy of *Plasmodium berghei*. *Exp. Parasitol.*, **25**: 283.
5. Bartlett, G.R. (1959). Phosphorous assay in column chromatography. *J. Biol. Chem.*, **234**: 466.
6. Basu-Mallik, K.C., D.K. Bhattacharya and N.C. Ganguly (1962). Studies on the enzyme activities of liver homogenate and plasma of albino rats infected with *P. berghei*. *Indian J. Med. Sci.*, **16**: 334.
7. Bergmeyer, H.U. and E. Benut (1963). Determination of glucose with glucose oxidase and peroxidase. In *Methods of Enzymatic Analysis*. Ed. H.U. Bergmeyer: 123.
8. Besey, O.A., O.H. Lowry and M.J. Brock (1946). A method for the rapid determination of alkaline phosphatase with five cubic millimeter of serum. *J. Biol. Chem.*, **164**: 321.
9. Buangener, W. (1965). The activity of nucleases and purine destroying enzymes in experimental *Plasmodium berghei* infection in rat. *Z. Tropenmed. Parasitol.*, **16**: 365.
10. Chatterji, A. and P.C. Sen Gupta (1957). Alkaline phosphatase and glycogen content of the liver in rats infected with *P. berghei*. *Bull. Calcutta Sch. Trop. Med.*, **5**: 169.
11. Chauhan, A., V.P.S. Chauhan and A.K. Sarkar (1981). Lipid peroxidation in various tissues of *Plasmodium berghei* infected mice. *I R C S Med. Sci. Libr. Compound*, **9**: 829.
12. Crane, R.K. and A. Sols (1953). The association of hexokinase with particulate fraction of brain and other tissue homogenates. *J. Biol. Chem.*, **203**: 273.
13. DeMoss, R.D. (1955). Glucose-6-phosphate and 6-phospho-gluconic dehydrogenases from *Leuconostoc mesenteroides*. In *Methods in Enzymology, v. 1*. Eds. S.P. Colowick and N.O. Kaplan. (Academic Press Inc., New York): 330.
14. Devakul, K. (1960). Sugar metabolism in malaria. *Trans. R. Soc. Trop. Med. Hyg.*, **54**: 87.
15. Devakul, K. and B.G. Maegraith (1958). Blood sugar and tissue glycogen in infections in *Macaca mulatta* with the Nuri strain of *Plasmodium knowlesi*. *Ann. Trop. Med. Parasitol.*, **52**: 366.
16. Fletcher, K.A. and B.G. Maegraith (1962). Glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities in erythrocytes of monkeys infected with *Plasmodium knowlesi*. *Nature*, **196**: 1316.
17. Folch, J., M. Lees and G.H. Sloane Stanley (1957). A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.*, **226**: 497.
18. Good, C.A., H. Kramer and M. Somogyi (1933). The determination of glycogen. *J. Biol. Chem.*, **100**: 485.
19. Gottfried, S.P. and B. Rosenberg (1973). Improved manual spectrophotometric procedure for determination of serum triglycerides. *Clin. Chem.*, **19**: 1077.
20. Herman, Y.F., R.A. Ward and R.H. Herman (1966). Stimulation of the utilization of 14C-glucose in chicken red blood cells infected with *Plasmodium gallinaceum*. *Am. J. Trop. Med. Hyg.*, **15**: 276.
21. Irie, M., T. Yamado and T. Ukita (1966). Purification and properties of whole pancreatic ribonuclease. *J. Biochem.*, **59**: 344.
22. Ishihara, T., M. Irie and T. Ukita (1967). Purification and properties of horse pancreatic ribonuclease. *J. Biol. Chem.*, **62**: 430.
23. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**: 265.
24. Maegraith, B.G. (1954). Some physiological and pathological processes in *Plasmodium berghei* infections in white rats. *Indian J. Malariol.*, **8**: 281.
25. Maegraith, B.G. (1968). Liver involvement in acute mammalian malaria with special reference to *Plasmodium knowlesi* malaria. *Adv. Parasitol.*, **6**: 189.
26. Maegraith, B.G., M.V. Riley and T. Deegan (1962). Changes in the metabolism of liver mitochondria of monkeys infected with *Plasmodium knowlesi* and their importance in pathogenesis of malaria. *Ann. Trop. Med. Parasitol.*, **56**: 483.
27. Marinetti, G.V. (1962). Chromatographic separation, identification and analysis of phospholipids. *J. Lipid. Res.*, **3**: 1.
28. Mercado, T.I. and T. von Brand (1954). Glycogen studies on white rats infected with *Plasmodium berghei*. *Exp. Parasitol.*, **3**: 259.
29. Montgomery, R. (1957). Determination of glycogen. *Arch. Biochem. Biophys.*, **67**: 378.
30. Ochoa, S. (1955). Malic dehydrogenase from pig heart. In *Methods in Enzymology, v. 1*. Eds. S.P. Colowick and N.O. Kaplan. (Academic Press Inc., New York): 735.
31. Rama Rao, R. (1978). Malaria infection and the nucleic acids of the chick. *J. Com. Dis.*, **10**: 165.
32. Rao, K.N., D. Subrahmanium and S. Prakash (1967). Studies on the phospholipids of mouse spleen on infection with *Plasmodium berghei*. *Indian. J. Biochem.*, **4**: 222.

33. Riley, M.V. and B.G. Maegraith (1962). Changes in the metabolism of liver mitochondria of mice infected with rapid acute *P. berghei* malaria. *Ann. Trop. Med. Parasitol.*, **56**: 473.

34. Riley, M.V. and T. Deegan (1960). The effect of *Plasmodium berghei* malaria on mouse liver mitochondria. *Biochem. J.*, **76**: 41.

35. Saxena, J.K., S. Ghatak and A.B. Sen (1981). *Plasmodium berghei*: Studies on host metabolism using *Mastomys natalensis* as experimental model. *Indian. J. Malariol.*, **18**: 80.

36. Schneider, W.C. (1945). Phosphorous compounds in animal tissues. *J. Biol. Chem.*, **161**: 293.

37. Sharma, R.P., O.P. Shukla, Chanan Singh and A.B. Sen (1979). Alterations in some biochemical parameters in mouse liver and spleen during infection with *Plasmodium berghei*. *Indian J. Med. Res.*, **69**: 944.

38. Sibley, J.A. and A.L. Lehninger (1949). Determination of aldolase in animal tissues. *J. Biol. Chem.*, **177**: 859.

39. Slater, E.C. and W.D. Bonner (1952). The effect of fluoride on the succinic oxidase system. *Biochem. J.*, **52**: 185.

40. Srivastava, I.K., N. Saxena, V.C. Pandey and G.P. Dutta (1982). Glucose-6-phosphate dehydrogenase in *Plasmodium knowlesi*. *Indian J. Malariol.*, **19**: 97.

41. Sutherland, L. and W.D. Wosilait (1956). The relationship of epinephrine and glucagon to liver phosphorylase. *J. Biol. Chem.*, **218**: 459.

42. Von Brand, T. and T.I. Mercado (1958). Quantitative and histochemical studies on liver lipids of rats infected with *Plasmodium berghei*. *Am. J. Hyg.*, **67**: 311.

43. Wotton, I.D.P. (1964). Enzyme in blood—Microanalysis. In *Medical Biochemistry*. 4th ed. (J. & A. Churchill Ltd., Lond.): 101.

44. Wroblewski, T. and J.S. LaDue (1955). Lactic dehydrogenase activity in blood. *Proc. Soc. Exp. Biol. Med.*, **90**: 210.

45. Wybenga, D.R., V.J. Pileggi, P.H. Dirstine and J. Digiorgio (1970). Direct manual determination of serum total cholesterol with a single stable reagent. *Clin. Chem.*, **16**: 980.

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An Evaluation of *Plasmodium cynomolgi bastianellii* and *Plasmodium knowlesi* Antigens in the Seroepidemiology of Human Malaria using Indirect Haemagglutination Test

AMAR NATH[1], INDRESH KUMAR SRIVASTAVA[1], RAGINI SINHA[1], S.S. AGARWAL[2] and G.P. DUTTA[1]

Comparison of *Plasmodium cynomolgi bastianellii* and *Plasmodium knowlesi* antigens in the indirect haemagglutination (IHA) test for malaria antibody revealed that at a cut off titre of 1 in 32, *P. cynomolgi* B antigen detects slightly more malaria cases than *P. knowlesi* antigen though the differences between the antigens were not significant. Using glutaraldehyde fixed tanned sheep erythrocytes sensitized with *P. cynomolgi* antigen, an IHA positivity of 84.82% (GMRT 237.6000), 12.90% (GMRT 4.136) and 1.53% (GMRT 3.445) was recorded in different groups i.e., slide positive malaria patients, patients of pyrexia of varied origin and normal healthy subjects respectively, whereas with *P. knowlesi* antigen the seropositivity was 77.67% (GMRT 154.100), 8.06% (GMRT 2.892) and 1.53% (GMRT 3.614) respectively, for these groups. With *P. cynomolgi* antigen, the GMRT of sera from patients with *P. falciparum* infection was 233.2 and with *P. vivax* infection 244.4, while with *P. knowlesi* antigen the GMRT of these sera were 174.6 and 127.9 respectively. Use of simian malaria parasite, particularly *P. cynomolgi* antigen should be given a further trial for seroepidemiological studies in *P. vivax* areas since this antigen has given higher GMRT.

INTRODUCTION

Serological tests for malaria antibody can provide valuable data for measuring the level of malaria endemicity in a given population, in delineating and characterizing new foci of malaria transmission and also in studying the malaria transmission patterns (Kagan *et al.*, 1969; Bruce-Chwatt, 1970). Indirect haemagglutination (IHA) test has been extensively used in the serodiagnosis and seroepidemiology of human malaria (Farshy and Kagan, 1972; Kagan, 1972, 1981; Meuwissen *et al.*, 1972). Inspite of the fact that the use of homologous antigens (*P. falciparum* or *P. vivax*) are known to increase both sensitivity and specificity of the IHA test (Meuwissen *et al.*, 1973; Mathews *et al.*, 1975; Molineaux and Gramiccia, 1980), limited number of investigators have used *in vitro* cultured *P. falciparum* antigen in IHA test for the seroepidemiological studies on malaria in India (Gupta *et al.*, 1981; Ray *et al.*, 1981, 1983). Previously several workers have successfully employed simian malaria antigen (*P. knowlesi*)

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[1]Division of Microbiology
Central Drug Research Institute, Lucknow-226001, India.
[2]Deptt. of Medicine
K.G. Medical College, Lucknow-226001, India.

which can be produced in bulk for serological studies on human malaria (Kagan *et al.*, 1969; Agarwal *et al.*, 1981, 1982). The present study was designed with a view to evaluate *P. cynomolgi bastianellii* (*P. cynomolgi* B) antigen in the seroepidemiology of human malaria.

MATERIAL AND METHODS

Antigen: Soluble *P. cynomolgi* B antigen used for sensitization of sheep erythrocytes was prepared from experimentally infected rhesus monkey according to the procedure described by Agarwal *et al.* (1981), except that monkey was bled when a schizont parasitaemia of 8–10% was attained. The schizont infected erythrocytes were separated using Ficoll-Conray 420 density gradient and these were then subjected to saponin lysis for half an hour, washed thrice with PBS (pH 7.2) and stored at −196°C in liquid nitrogen. On the day of actual test, an aliquot of the schizont antigen was disrupted by means of ultrasonication and the soluble fraction was used as a source of antigen (WHO, 1974). The same batch of antigen was used throughout the studies reported in this paper.

Sera: Serum samples from three groups of subjects were included in this study. Group–I consisted of one hundred and twelve serum samples from slide positive malaria cases collected from the field at Mathura, Mirzapur and Lucknow districts of Uttar Pradesh which were tested by the IHA test for the presence of antimalarial antibodies. Out of these, 67 patients harboured *P. falciparum* infection while 45 cases were of *P. vivax* infection (Table 1). Control groups comprised of sera samples from 62 patients of pyrexia of varied origin (Group–II), and 130 normal healthy subjects (Group–III). All the controls included in the study were slide negative for malaria. The sera were stored at −20°C and inactivated at 56°C for 30 mins. prior to use.

IHA Test: IHA test was performed using permanent lucite microtitre plates with 96-U-shaped wells (Cooke No. I–220-6 Dynatech Lab., Singapore). Determination of optimal sensitizing dose of antigen, the procedure for sensitization of the glutaraldehyde fixed and tanned sheep erythrocytes and interpretation of results were largely carried out as described in WHO memorandum (WHO, 1974) and reported in our earlier study (Agarwal *et al.*, 1982). A known positive malaria immune serum from monkey which gave a titre of 1 in 1024 and known negative serum which gave titre of 1 in 4 or less was used in each series of the test. Absorption for heterophile antibodies with sheep RBC as recommended by WHO (1974), was carried out with selected sera, but no significant difference between absorbed and unabsorbed sera was found.

RESULTS

Results of the IHA test with *P. cynomolgi* B antigen are presented in Table 2. At a cut off titre of 1:32 suggestive of malaria, 84.82% of the

Table 1. Locality-wise distribution of malaria patients whose sera were used for serology

Locality	No. of patients	No. with infection	
		P. falciparum	*P. vivax*
Ral (Mathura, U.P.)	41	38	3
Mant (Mathura, U.P.)	34	22	12
Shaktinagar (Mirzapur, U.P.)	26	7	19
Lucknow (U.P.)	11	Nil	11
Total	112	67	45

Table 2. Reciprocal IHA titres among different groups using *P. cynomolgi* B antigen

Group	GMRT	No. of patients showing reciprocal IHA titre								
		4096	≥2048	≥1024	≥512	≥256	≥128	≥64	≥32	≥16
I. Malaria patients (n = 112)	237.6	8 (7.14)	19 (16.96)	39 (34.82)	57 (50.89)	65 (58.03)	73 (65.17)	84 (75.0)	95 (84.82)	108 (96.42)
(i) *P. falciparum* infected (n = 67)	233.2	4 (5.97)	11 (16.41)	26 (38.80)	36 (53.73)	40 (59.70)	44 (65.62)	47 (70.14)	54 (80.59)	64 (95.92)
(ii) *P. vivax* infected (n = 45)	244.4	4 (8.88)	8 (17.77)	13 (28.88)	21 (46.66)	25 (55.55)	29 (64.44)	37 (82.22)	41 (91.11)	44 (97.97)
II. Pyrexia of varied origin (n = 62)	4.136	Nil	Nil	Nil	Nil	Nil	Nil	1 (1.61)	8 (12.90)	17 (27.41)
III. Normal Healthy Subjects (n = 130)	3.445	Nil	Nil	Nil	Nil	Nil	Nil	1 (0.76)	2 (1.53)	13 (10.0)

GMRT = Geometric mean reciprocal titre; Figures in parentheses indicate the cumulative percentages.

Significance of seropositivity between different groups at cut off titre of 1 in 32.

Normal vs Pyrexia $p<0.01$.

Normal vs *P. vivax* infected $p<0.01$.

Normal vs *P. falciparum* infected $p<0.01$.

Normal vs Total malaria patients $p<0.01$.

malaria patients (Group-I) yielded positive IHA response, while only 12.90% of the cases of pyrexia (Group-II) and 1.53% normal healthy subjects (Group-III) gave a positive test. Seropositivity was 80.59% among cases of *P. falciparum* infection and 91.11% in cases of *P. vivax* infection. The geometrical mean reciprocal titres (GMRT) for Group-I, Group-II and Group-III were found to be 237.6, 4.136 and 3.445, respectively. The GMRT was 233.2 and 244.4 among cases of *P. falciparum* and *P. vivax* infection respectively.

Table 3 summarizes the results of the IHA test with *P. knowlesi* antigen. At a diagnostic titre of 1:32, 77.67% positivity was obtained in Group-I, 8.06% in Group-II and 1.53% in Group-III. Using this antigen a positivity of 76.10% was obtained in *P. falciparum* infected cases while in *P. vivax* cases the positivity was 80.00%. The geometrical mean reciprocal titres of Groups I to III were 154.1, 2.892 and 3.614 respectively. The GMRT was 174.6 for *P. falciparum* infection and 127.9 for *P. vivax* infection.

DISCUSSION

A limited number of investigators have used *P. falciparum* antigen in India for serological studies on malaria. Ray *et al.* (1981, 1983 a, b) have used *P. falciparum* antigen for ELISA, IFA and IHA tests and Gupta *et al.* (1979) employed *P. falciparum* antigen for IHA test. The ELISA technique with *P. falciparum* antigen was also applied by Mahajan *et al.* (1981) and Dutta *et al.* (1982) in the seroepidemiology of human malaria. Earlier studies carried out by Ambroise-Thomas *et al.* (1976), Collins and Skinner (1972), Coudert *et al.* (1965) and Kuvin *et al.* (1967) revealed that *P. cynomolgi* B antigen could be successfully employed in the IFA test for the serodiagnosis of human malaria. This is the first report on the successful use of *P. cynomolgi* B antigen in IHA test for seroepidemiology and our results have shown an overall seropositivity of 84.82% among malaria patients (80.59% in cases of *P. falciparum* infection and 91.11% in cases of *P. vivax* infection). These observations are in close

Table 3. Reciprocal IHA titres among different groups using *P. knowlesi antigen**

Group	GMRT	No. of cases showing reciprocal IHA titre of 1:32 (% cases)
I. Malaria patients (n = 112)	154.1	87 (77.67%)
(i) *P. falciparum* infected (n = 67)	174.6	51 (76.10%)
(ii) *P. vivax* infected (n = 45)	127.9	36 (80.00%)
II. Pyrexia of varied origin (n = 62)	2.892	5 (8.06%)
III. Normal Healthy Subjects (n = 130)	3.614	2 (1.53%)

GMRT = Geometrical mean reciprocal titre.

**P. knowlesi* data summarized from Agarwal *et al.* (1982) and given for comparison.

Significance of seropositivity between different groups at cut off titre 1:32.

Normal vs Pyrexia $p<0.01$.

Normal vs *P. vivax* infected $p<0.01$.

Normal vs *P. falciparum* infected $p<0.01$.

Normal vs Total malaria patients $p<0.01$.

Table 4. Correlation of antigenic reactivity of *P. cynomolgi* and *P. knowlesi* antigen in the IHA test for human malaria

Infection	No.	Number of patients			
		Pc+ Pk+	Pc+ Pk−	Pc− Pk+	Pc− Pk−
P. falciparum	67	48	6	3	10
P. vivax	45	36	5	Nil	4

Pc= *P. cynomolgi*; Pk= *P. knowlesi*.
For *P. knowlesi* data refer to Agarwal *et al.* (1982).
Significance between the sensitivity of antigens, *P. knowlesi* vs *P. cynomolgi* : Not significant

agreement with those of Agarwal *et al.* (1983) and Voller and Bruce-Chwatt (1968) who reported 90.72 and 92% positivity respectively, in the IFA test using *P. cynomolgi* antigen.

P. cynomolgi B was found to be an ideal antigen for the detection of *P. vivax* infection as compared to *P. knowlesi* antigen, as sera of 91.11% slide positive *P. vivax* malaria cases yielded positive IHA response with a very high GMRT of 244.4, in contrast to a positivity rate of 80% with a lower GMRT of 127.9 obtained with *P. knowlesi* antigen. These observations are in accordance with those of Desowitz *et al.* (1966), Stein and Desowitz (1964) and WHO (1974) who also recommended the use of *P. cynomolgi* antigen in detection of *P. vivax* infection.

P. cynomolgi antigen detected 6 more cases of *P. falciparum* infection which were missed by *P. knowlesi* antigen while *P. knowlesi* detected only 3 cases in addition that were missed by *P. cynomolgi* antigen. Not only this, *P. cynomolgi* detected 5 more cases of *P. vivax* infection than those detected by *P. knowlesi* antigen (Table 4). Our results with IHA test strengthen the observation made earlier by Kuvin and Voller (1963), Mamtani *et al.* (1979) and Voller and Schindler (1967) who recommended the specific use of *P. cynomolgi* antigen for the detection of malarial antibodies.

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REFERENCES

1. Agarwal, S.S., Amar Nath, Pawan Sharma, I.K. Srivastava, S.R. Dwivedi and G.P. Dutta (1983). Comparative evaluation of *P. knowlesi* and *P. cynomolgi* antigen in the indirect fluorescent antibody test for human mala..a. *Indian J. Med. Res.*, 77: 616-622.
2. Agarwal, S.S., Amar Nath, Pawan Sharma, I.K. Srivastava, S.R. Dwivedi, R.L. Yadava and G.P. Dutta (1982). Seroepidemiology of human malaria: Indirect haemagglutination test using the *Plasmodium knowlesi* antigen. *Indian J. Malariol.*, 19: 21-25.
3. Agarwal, S.S., Pawan Sharma, Amar Nath, I.K. Srivastava, Jagdish Rai and G.P. Dutta (1981). Indirect haemagglutination and indirect fluorescent antibody tests for human malaria using *P. knowlesi* antigen. *Indian J. Malariol.*, 18: 67-72.

4. Ambroise-Thomas, P., W.H. Wernsdorfer, B. Grab, J. Cullen and P. Bertagna (1976). A seroepidemiological study of malaria in Tunisia. *Bull. WHO*, **54**: 355-367.
5. Bruce-Chwatt, L.J. (1970). Seroepidemiology of malaria. *J. Parasitol.*, **56**: 552-555.
6. Collins, W.E. and J.C. Skinner (1972). The indirect fluorescent antibody test for malaria. *Am. J. Trop. Med. Hyg.*, **21**: 690-695.
7. Coudert, J., J.P. Garin, P. Ambroise-Thomas, P. Saliov and M. Minjat (1965). Utilisation de *P. cynomolgi bastianellii* dans de serodiagnostic. *Bull. Soc. Path. Exot.*, **58**: 630.
8. Desowitz, R.S., J.J. Saave and B. Stein (1966). The application of indirect haemagglutination antibody test in recent studies on the immunoepidemiology of human malaria and the immune response in experimental malaria. *Milit. Med.*, **131** (Suppl.): 115.
9. Dutta, G.P., I.K. Srivastava, Pawan Sharma, Amar Nath and S.S. Agarwal (1982). Enzyme linked immunosorbent test for diagnosis of human malaria. *Indian J. Malariol.*, **19**: 33-37.
10. Farshy, D.C. and I.G. Kagan (1972). Use of stable sensitized cells in indirect microhaemagglutination test for malaria. *Am. J. Trop. Med. Hyg.*, **21**: 868-872.
11. Gupta, M.M., P.G. Jayaprakash, P. Nirmala, M.J. Sebastian and P. Bhat (1979). A preliminary report on serological studies on malaria in the rural population of Karnataka State. *Indian J. Med. Res.*, **70** (Suppl.): 62-66.
12. Kagan, I.G. (1972). Evaluation of the indirect haemagglutination test as an epidemiologic technique for malaria. *Am. J. Trop. Med. Hyg.*, **21**: 683-689.
13. Kagan, I.G. (1981). The indirect haemagglutination and latex test for malaria serodiagnosis. (Unpublished document). *WHO Mal.*, **81**: 954.
14. Kagan, I.G., H. Mathews and A.J. Sulzer (1969). The serology of malaria: recent publications. *Bull. N. Y. Acad. Med.*, **45**: 1027-1042.
15. Kuvin, S.F. and A. Voller (1963). Malaria antibody titres of West Africans in Britain. *Br. Med. J.*, **2**: 477-479.
16. Kuvin, S.F., J.E. Tobic, C.B. Evans, G.R. Coatneyi and P.G. Contacos (1962). Antibody production in human malaria as determined by the fluorescent antibody technique. *Science*, **135**: 1130-1131.
17. Mahajan, R.C., N.K. Ganguly, M. Thadani, Nivanjit Gill and R.E. Chandnani (1981). Comparison of ELISA with indirect haemagglutination and indirect fluorescent antibody test in malaria. *Indian J. Med. Res.*, **73**: 874-879.
18. Mamtani, R., B. Ravindran, K. Bagchi, P. Malhotra and Q.Z. Hussain (1979). A serological survey for malaria in a rural community near Delhi. *Indian J. Med. Res.*, **70**: 199-205.
19. Mathews, H.M., J.A. Frieds and I.G. Kagan (1975). The indirect haemagglutination test for malaria evaluation of antigen prepared from *P. falciparum* and *P. vivax*. *Am. J. Trop. Med. Hyg.*, **24**: 417-422.
20. Meuwissen, J.H.E. Th., A.D.E.M. Leeuwenberg and G.E. Molenkamp (1972). Studies on the various aspects of indirect haemagglutination test for malaria. *Bull. WHO*, **46**: 771-782.
21. Meuwissen, J.H.E. Th., A.D.E.M. Leeuwenberg, W.N. Doesberg and J.A.H. van Druten (1973). Application of indirect haemagglutination antibody test for malaria. Reproducibility with *P. falciparum* sensitized cells. *Bull. WHO*, **49**: 317-319.
22. Molineaux, L. and G. Gramiccia (1980). *The Garki Project: Research on the epidemiology and control of malaria in the Sudan Savanna of West Africa*. WHO, Geneva : 311.
23. Ray, K., A.N. Raichowdhuri, R.S. Sharma, Arvind Rai, D.S. Choudhury and H.O. Lobel (1981). Indirect haemagglutinating malaria antibodies in fever cases from a rural community in Alwar District, Rajasthan. *Indian J. Med. Res.*, **73** (Suppl.): 78-81.
24. Ray, K., M.C. Sharma, C.A. Sivaraman and A.N. Raichowdhuri (1983a). Evaluation of IHA test using *P. falciparum* from *in vitro* culture and its comparison with IIF and ELISA. *Indian J. Med. Res.*, **78**: 624-630.
25. Ray, K., M.C. Sharma, C.A. Sivaraman and A.N. Raichowdhuri (1983b). *In vitro* culture as a source of *P. falciparum* antigen in micro-ELISA in malaria. *Indian J. Med. Res.*, **78**: 205-209.
26. Stein, B. and R.S. Desowitz (1964). The measurement of antibody in human malaria by a formolised tanned sheep cell haemagglutination test. *Bull. WHO*, **30**: 45-49.
27. Voller, A. and L.J. Bruce-Chwatt (1968). Serological malaria surveys in Nigeria. *Bull. WHO*, **39**: 883-897.
28. Voller, A. and R. Schindler (1967). An evaluation of complement fixation and immunofluorescent test with a simian malaria parasite antigen, in a study of malaria antibody levels in a population in malaria endemic areas. *Bull. WHO*, **37**: 675-678.
29. WHO (1974). Serological testing in malaria. *Bull. WHO*, **50**: 527-535.

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A Comparison of Lignin Test and Filter Paper Spot Test for the Screening of the Long acting Sulfonamides in Urine

R.G. ROY[1] and S. BALAKRISHNAN[1]

Before undertaking the *in vivo* 4 - aminoquinoline or sulfa/ pyrimethamine sensitivity tests in *P. falciparum*, urine examination is essential to exclude the administration of such drugs in the recent past. A comparison was made of two screening tests—Lignin test and paper spot test–for detection of long acting sulfonamides in urine. Specimens of urine from 21 subjects receiving sulfadoxine and pyrimethamine and 17 receiving sulfalene and pyrimethamine in a single dose of 1000 mg (sulfadoxine/sulfalene) were screened for the presence of the drug for 10 consecutive days. Paper spot test could detect the sulfa drug upto 10 days and gave positive reaction when the concentration was 5µg or more per ml. The Lignin test, on the other hand, gave positive results only upto 4 days and could detect the drug only at concentrations of 20µg and above per ml. Paper spot test would therefore,be better suited for field use for the *in vivo* urine tests for this antimalarial drug.

INTRODUCTION

The problem of drug resistance to 4-aminoquinolines in *P. falciparum* is causing a great global concern. *In vivo* as well as *in vitro* tests recommended by WHO are now widely used in different parts of the world for monitoring the susceptibility status of *P. falciparum* (WHO, 1973). Resistance to long acting sulfonamide in combination with pyrimethamine, used as an alternative to the 4-aminoquinolines, has also been reported from some countries recently (Wernsdorfer, 1983).

Before selecting the patients it is very necessary to ensure that they have not received any 4-aminoquinolines, quinines or tetracycline during the previous 14 days, while drug free intervals of 4 weeks and 6 weeks are required in case of sulfadoxine pyrimethamine and mefloquine respectively (WHO, 1982). For this purpose, the screening of the urine is carried out as a routine for the presence of 4-aminoquinolines and sulfa drugs. It is also essential that the evidence of absorption, after the drug ingestion, is obtained by such tests. Currently, Lignin test is being widely used for the detection of sulfa drugs in the urine (Gradwohl, 1963).

The application of a paper spot test for screening dapsone (DDS) in urine employing a modified Ehrlich reagent was reported by Balakrishnan (1968). Its usefulness and feasibility in field situations has been clearly demonstrated (Noordeen and Balakrishnan, 1972; Ashok

Accepted for publication: 20 October 1984.
[1]Central Leprosy Teaching & Research Institute
Chengalpattu 603001, India

Kumar and Balakrishnan, 1982). During the standardisation of the test, it was observed that the sulfonamides also gave a colour similar to dapsone, which is understandable, as sulfones and sulfonamides belong to the same family. It was therefore, thought that a comparison of the paper spot test and the Lignin test for the detection of long acting sulfonamides, used as antimalarials, would be interesting and useful.

The present communication summarises the findings of the 2 screening tests on urine specimens obtained from subjects receiving either sulfalene and pyrimethamine (Metakelfin®) or sulfadoxine and pyrimethamine (Rimodar® Fansidar®).

MATERIAL AND METHODS

Seven healthy volunteers and 31 leprosy patients formed the subject of the study. Two tablets in a single dose of Rimodar® were administered to 21 subjects and 2 tablets in a single dose of Metakelfin® were administered to 17 subjects. In the case of leprosy patients, all drugs including DDS were withdrawn for 7 days before administration of these tablets, and for the next 10 days, DDS and other drugs were not given.

Random urine specimens were collected 24 hrs after the administration of the drug and daily for the following nine days.

Lignin test: The screening for sulfonamides by the Lignin test was performed as described below:

Reagent : 25% Hydrochloric acid

One or two drops of urine were put on the blank strip of newspaper. On this, one drop of hydrochloric acid solution was added. An intense orange colour indicated the presence of sulfonamides. A drop of HCl solution on the blank paper produced a straw yellow colour (blank colour).

Paper spot test: The filter paper spot test (Balakrishnan, 1968) was performed, as briefly outlined below:

Modified Ehrlich Reagent of Castro *et al.* (1963)

4- dimethyl amino benzaldehyde	0.2 gm
Oxalic acid	1.0 gm
Nacconol	0.1 gm
Redistilled alcohol	100 ml

The reagent was filtered 24 hrs after preparation to remove excess nacconol. Whatman 3 mm filter paper strips of 1 cm width were dipped in the reagent and allowed to air-dry. One drop of the urine containing sulfonamides gave a bright yellow colour immediately. (The appearance of a greenish yellow ring, sometime later, at the periphery is due to urea). The strip was subsequently dipped into 0.1 M citric acid solution to differentiate the colour due to urea (which disappears) from that of the sulfonamides.

The relative sensitivities of the two screening tests for the detection of Metakelfin® as well as Rimodar®, were compared, employing a range of concentrations from 5 to 100 µg per ml.

RESULTS

The findings on the sensitivity of the screening tests in respect of the 2 drugs are given in the Table 1 while the results of the two screening tests for the two drugs in urine upto 10 days after administration are shown in Table 2.

Table 1. Sensitivity of Lignin test and Spot test in urine

Concentration in μg/ml	Metakelfin ^{R}		Rimodar ^{R}	
	Spot test	Lignin test	Spot test	Lignin test
100	+	+	+*	+
50	+	+	+	+
20	+	±	+	±
10	+	-	+	-
5	+		+	-

Note. Below 5 μg/ml, the tests were not +ve for either of the drugs.

Table 2. Duration of Positivity by Lignin and Spot test

Screening test	Total No. of subjects	Metakelfin ^{R} +ve upto days			Total No. of subjects	Rimodar ^{R} +ve upto days		
		8	9	10		8	9	10
Spot test with Ehrlich reagent	17	13 (76%)	2 (12%)	2 (12%)	21	17 (81%)	3 (14%)	1 (5%)
Lignin test	17	4	6		21	4	6	
		15 (88%)	2 (12%)			18 (86%)	3 (14%)	

DISCUSSION

Long acting sulfonamides have established a place in the chemotherapy of malaria, particularly in cases of proved resistance to chloroquine. It appears almost certain that the urine tests for the presence or absence of the long acting sulfa drugs will be carried out more widely as a part of the sensitivity test for finding out the resistance status to these drugs as a primary objective, in addition to its use for the chloroquine resistance studies.

Consequently, monitoring these drugs in body fluids assumes a great significance. Lignin test has been widely used for the detection of sulfonamide in urine. The experience with the spot test employing a modified Ehrlich reagent in this institution for dapsone, prompted a comparison of this test with Lignin test. The relative advantages and disadvantages of these two tests are shown in Table 3.

The findings presented in Tables 1, 2 & 3 clearly indicate the superiority of the spot test over the Lignin test in regard to its sensitivity. The slightly higher percentage of positive spot tests in the case of subjects receiving Rimodar^{R} is in keeping with a much longer half life of this drug (150–200 hrs) as compared to Metakelfin^{R} (65 hrs) (Bruce-Chwatt, 1980). In view of these observations, the spot test appears to be better suited for the screening of the long acting antimalarials like sulfadoxine and sulfalene.

Table 3. Advantages and Disadvantages of Lignin test and Spot test

Lignin test

	Advantages		Disadvantages
(a)	Only chemical required is HCl in addition to paper towel or news paper.	(a)	Appearance of a mild yellow colour without the presence of sulfonamide (blank colour) i.e., false positivity is a likelihood.
(b)	Simple field test	(b)	Low sensitivity (20 μg/ml and above) after a single dose of 1000 mg base. The test tends to become negative by the 5th day.
(c)	Cheap and operationally feasible in the field.	(c)	Handling of a relatively concentrated acid is involved.
		(d)	Texture of the paper gives diffuse spots.

Spot test with Ehrlich's reagent

	Advantages		Disadvantages
(a)	High sensitivity i.e., 5 μg and above	(a)	Use of a special quality paper i.e., Whatman 3 mm thick. (This should not be a difficulty).
(b)	After a single dose of 1000 mg base of long acting sulfa (sulfalene, sulfadoxine), the test is positive for 8 days in about 80% of cases.	(b)	Reagent has to be prepared (Technique is not difficult); nacconol, a constituent of the reagent may not be available readily in some areas, but the reagent can be prepared without this substance and the test carried out.
(c)	Clear differentiation between positives and negatives.		
(d)	Cheap and operationally feasible in the field.		
(e)	No handling of any strong acid is involved.		
(f)	Use of a special quality filter paper gives discrete spots.		

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REFERENCES

1. Ashok Kumar and S. Balakrishnan (1982). Monitoring the regularity of self-administration of dapsone by leprosy patients. *Lep. in India*, **54**: 665.
2. Balakrishnan, S. (1968). Application of a spot test for detection of DDS in urine. *Lep. in India*, **40**: 1-5.
3. Bruce-Chwatt, L.J. (1980). *Essential Malariology*. 1st ed. (William Heineman Medical Books Ltd., London): 182-183.
4. De Castro, J., S.M. de Almeida and N.J. Nogueira de Castro (1963). Control of absorption of antileprosy drugs in public health campaign. Summary. *Proc. 7th Int. Cong. Drop. Med. Malar.*, **2**: 53.
5. Gradwohl (1963). *Clinical Laboratory Methods and diagnosis*, v.1. 6th ed. Eds. Frankel and Reitman. (C.V. Mosby, St. Louis): 421.
6. Noordeen, S.K. and S. Balakrishnan (1972). Spot test for DDS (diaminodiphenyl Sulphone) in urine under field conditions. *Indian J. Med. Res.*, **60**: 367-371.
7. Wernsdorfer, W. (1983). Urgent efforts needed to combat drug resistant malaria. *WHO Chronicle*, **37**: 11-13.
8. WHO (1973). Chemotherapy of Malaria and Resistance to Antimalarials. *Tech. Rep. Ser. (WHO)*, **529**: 30-36.
9. WHO (1982). David Payne. Practical aspects of the *in vivo* testing for sensitivity of human *Plasmodium* spp. to antimalarials. Mal/82, **988**: 4.

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Effect of Malathion Spraying on Four Anopheline Species and the Development of Resistance in *A. stephensi* in Mandora, Haryana

SARALA K. SUBBARAO[1], V.P. SHARMA[1], K. VASANTHA[1] and T. ADAK[1]

Densities of *A. annularis*, *A.culicifacies*, *A. stephensi* and *A. subpictus* were monitored in village Mandora, Haryana, before and after malathion spraying. The malathion spraying interrupted the transmission of malaria and decimated the *A. annularis*, *A. culicifacies* and *A. subpictus* populations. The spraying was initially effective for *A. stephensi* but later there was a gradual build up of the populations, suggesting the ineffectiveness of the spray in the control. Bioassay tests confirmed that the resistance precipitated at a rapid rate in *A. stephensi*.

INTRODUCTION

In Mandora and several other villages of Haryana state, *A. culicifacies* is the principal vector of malaria. In these villages, yearly 2 to 3 cycles of HCH was sprayed at the rate of 200 mg/m^2 to control the disease. Epidemiological studies during 1980 and 1981 revealed that HCH spraying had no impact on the density of anophelines resulting in uninterrupted transmission of malaria. Several specimens of *A. culicifacies* were incriminated during an epidemic of falciparum malaria in these villages (Choudhury and Ghosh, 1982; Sharma *et al.*, 1983). The epidemic was controlled by malathion spraying at the rate of 2g/m^2 by the National Malaria Eradication Programme in 1982.

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[1]Malaria Research Centre (ICMR)
22 Sham Nath Marg, Delhi-110054, India.

In Mandora village, before spraying, *A. culicifacies* populations were being monitored for species composition (Subbarao *et al.*, 1980 and 1983) and densities of other anopheline species were also monitored. After spraying began in this village, monitoring of densities of four commonly encountered anophelines viz., *A. annularis*, *A. culicifacies*, *A. stephensi* and *A. subpictus* continued for a period of two years to study the impact of malathion spraying. Further, susceptibility tests of *A. stephensi* populations were carried out and the results of these studies are reported in this paper.

MATERIAL AND METHODS

The village Mandora is situated in Sonepat district, Haryana, approximately 38 km North-West of the Malaria Research Centre. For collection of adult mosquitoes, five sites were selected which included both human

dwellings and cattle sheds. The collections were made using a suction tube at an interval of about 15 days. Adult density data were pooled for the month and the density per man hour (mhd) was calculated. Malathion was sprayed in March, May and August of 1982, in March and June of 1983 and in April of 1984. Pre-spray data from October, 1981 to March, 1982 and post spray data from April, 1982 to October, 1983 were collected.

Insecticide susceptibility tests were carried out in the laboratory maintained at 27° ± 1°C and 60-80% RH. Adult female mosquitoes were collected from the field and transported to the laboratory for this purpose. When the collection of females from the field was not adequate, the females were held in the laboratory to lay eggs and their progeny were tested. On a few occasions, larvae were also collected from the field and reared in the laboratory to test the females. All mosquitoes were exposed to 5% malathion impregnated papers for one hour and mortality recorded after 24 hrs (WHO, 1975).

RESULTS AND DISCUSSION

The anopheline fauna of the village consists of four commonly found anophelines viz., *A. annularis*, *A. culicifacies*, *A. stephensi* and *A. subpictus* and occasionally *A.* nigerrimus and *A. pulcherimus*. It may be mentioned here that the majority of the *A. culicifacies* populations in this village were of species A (Subbarao *et al.*; 1980, 1983), and *A. stephensi* populations were of var. *mysorensis* and intermediate type (Subbarao *et al.*, 1984). The resistance status of *A. culicifacies* and *A. stephensi* to DDT and HCH ranged from 60-100% and 90-100% respectively in Mandora as well as in other villages around Delhi.

Monitoring of mosquito densities from October, 1981 to March, 1982 revealed that the peak densities of *A. culicifacies* (19.5 mhd) and *A. subpictus* (31.0 mhd) occurred in October, *A. annularis* (19.3 mhd) in December and *A. stephensi* (20.8 mhd) in February. During winter months, *A. subpictus* almost disappeared while density of *A. culicifacies* was reduced to low levels i.e., 5 per man hour (Fig. 1).

Malathion was sprayed for the first time in March, 1982. Spraying greatly reduced *A. culicifacies*, *A. subpictus* and *A. annularis* populations. Second round of spraying in May further reduced the remaining populations to almost negligible numbers (Fig. 1). Third round of spraying in August prevented the build up of mosquito populations and *A. culicifacies* was totally absent till August, 1983 and only 2 specimens, one each in late August and September were collected which were identified as species A. A few specimens, of *A. annularis* were found in April, 1983.

The first round of malathion spraying drastically reduced *A. stephensi* populations, but the remaining mosquitoes multiplied to high numbers. The second round of spraying in May had no effect on *A. stephensi* densities, rather, there was considerable increase in man hour density to as high as 25 in June. There were heavy rains in July and *A. stephensi* densities dropped to 5.2. Spraying in August reduced *A. stephensi* to low numbers and populations stayed at that level during the winter months. Spraying in March, 1983 had no effect on *A. stephensi* populations and in June, the densities reached 23.2 comparable to the man hour densities in June, 1982. In July, a low density of 8 was recorded which may have been either due to spraying (June, 30) or due to rains or the combined effect of both.

It is notable that five rounds of malathion spraying could not reduce *A. stephensi* populations to low levels as observed for other anophelines, *A. culicifacies*, *A. subpictus* and *A. annularis*. During the optimum weather conditions such as the one that existed in June, 1982 and 1983, *A. stephensi* populations multiplied even under the pressure of malathion spray. There was, therefore, direct evidence of

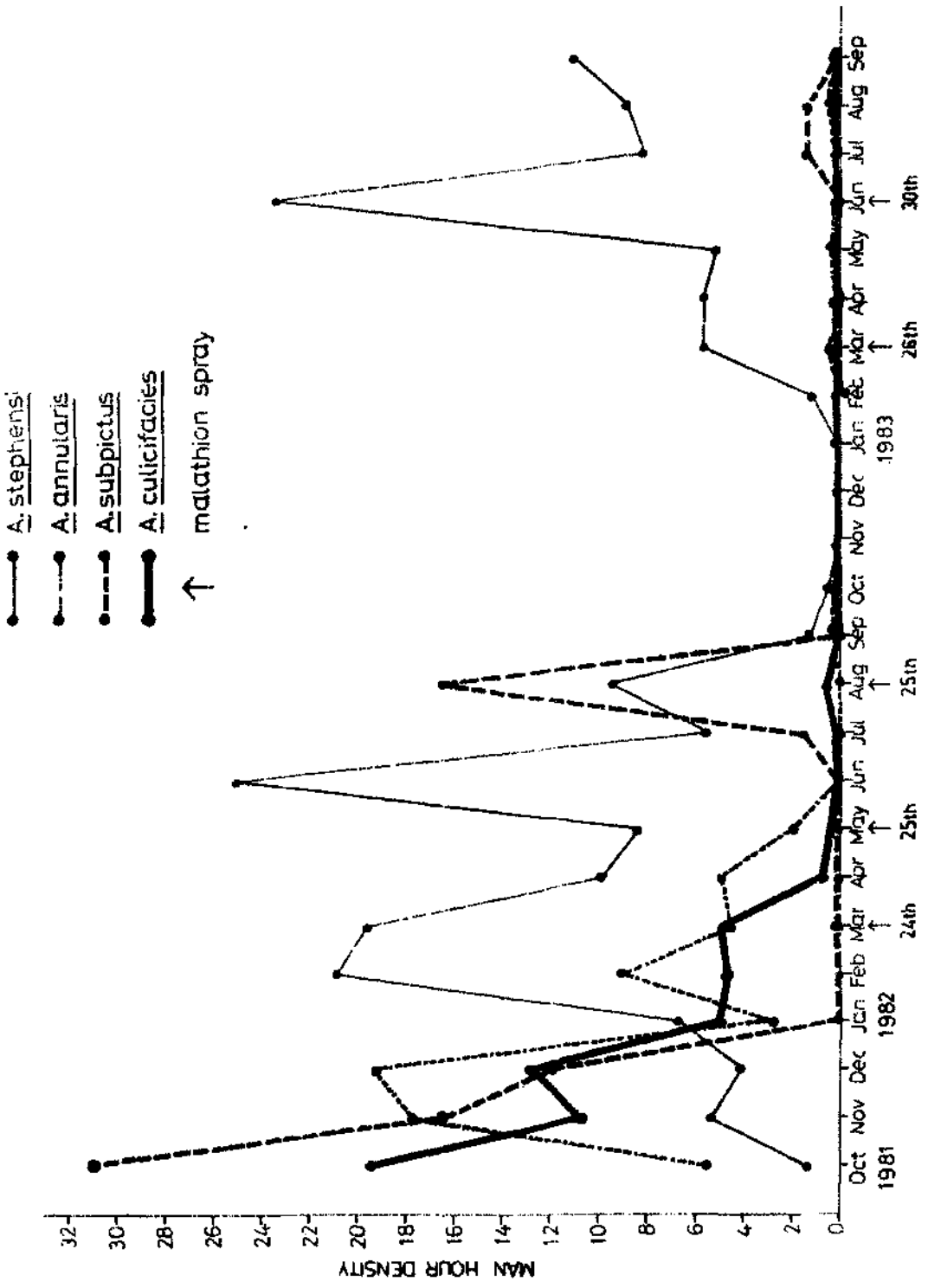

Fig. 1. Effect of malathion spraying on anopheline species in Mandora, Haryana.

the ineffectiveness of malathion spraying in the control of *A. stephensi*.

Bioassay tests were carried out exposing *A. stephensi* adults to 5% malathion-impregnated papers at various time intervals (Table 1). Results revealed that 5 weeks after the first round of malathion spraying there was 62% mortality in the field-collected *A. stephensi*. The mortality ranged between 0-38% after the fourth round of spraying and in April, 1984 i.e., 286 days after the fifth round of spray and just before the sixth round of spray, it was about 76%. After the sixth round of spraying (April 19, 1984) the mortality ranged between 46-62% in the susceptibility tests. The laboratory tests and field populations clearly suggest that there was a rapid build up of physiological resistance in *A. stephensi*, while the other anophelines viz., *A. annularis*, *A. culicifacies* and *A. subpictus* remained fully susceptible to malathion.

It may be pointed out that the first instance of malathion resistance in *A. culicifacies* was reported from Gujarat in 1973 (Rajagopal, 1971). The authors are not aware of any report on malathion resistance in *A. stephensi* in India and this may probably be the first such instance. In Bandar Abbas, Iran, malathion was sprayed from 1964 onwards at the rate of 2g/m^2 and 2-3 cycles/year. Even after continuous spraying, *A. stephensi* var. *mysorensis* remained susceptible for 10 years and only in 1975 i.e., after 26 rounds of spray, resistance was noted in this species (Manouchehri *et al.*, 1974, 1975). In Pakistan, this species developed resistance within three years of malathion spraying (Rathor and Toqir, 1980). The rapid evolution of resistance to malathion in Pakistan and in Mandora, while its delayed appearance in Bandar Abbas, Iran, suggests the possibility of different backgrounds of the two populations. Resistant gene is generally assumed to be at a mutation frequency of 1×10^{-5}. However, occasionally R genes may be found in polymorphic form as was the case for dieldrin resistance in *A. gambiae* (Brown and Pal, 1971). There is a possibility of malathion resistance being in polymorphic form in Pakistan and Mandora. But the LT_{50} values for *A. stephensi* and *A. culicifacies* with 5% malathion-impregnated papers were not significantly different in the unsprayed villages around Delhi. The values were 24 to 26.5 mins for *A. stephensi*; 15 to 18 mins for *A. culicifacies* and 25 mins for *A. subpictus*. Similar results were reported from Pakistan (Rathor and Toqir, 1980). They further

Table 1. Malathion susceptibility data of *A. stephensi* from Mandora, Haryana

Date of spray	Time of mosquito collection after the last spray	No. treated	% mortality	Remarks
1st Spray 24.3.82	37 days	29 (2)	62.0	Adult field collection
2nd Spray– 25.5.82				
3rd Spray– 25.8.82	199 days	32 (1)	9.3	Adult field collection
4th Spray– 26.3.83	17 days	100 (4)	28.0	Larval collection
	33 days	96 (4)	0	Adult field collection
	94 days	24 (1)	37.5	*do*
	94 days	75 (3)	21.3	F_1 adults
5th Spray 30.6.83	39 days	100 (4)	34.0	F_1 adults
	286 days	60 (3)	76.7	F_1 adults
6th Spray 19.4.84	8 days	30 (1)	46.6	F_1 adults
	21 days	108 (5)	62.0	F_1 adults

Parentheses indicate no. of replicates.

reported that immediately after two rounds of spray the LT_{90} values for *A. stephensi* increased 10-fold while it remained the same for *A. culicifacies*. This suggests that rapid build up of resistance in *A. stephensi* may not be due to the existence of R gene in polymorphic form but may be due to some other genetic factors. Malathion resistance in *A. stephensi* is monofactorial and codominant (Rathor and Toqir 1981; Hemingway, 1983) and this may have been one of the factors contributing to the rapid build up of resistance in the field populations. However, the reasons for delayed appearance of resistance in *A. stephensi* in Iran are not known and need to be examined.

During the 1981 epidemic, several specimens of *A. culicifacies* were found with sporozoites (83 out of 9124 dissected) but of 577 *A. stephensi* dissected none was incriminated (MRC Annual Report, 1981). Spraying of malathion in 1982 and 1983 decimated *A. culicifacies* populations and completely interrupted malaria transmission, though high densities of *A. stephensi* were present.

A. stephensi is a known vector of malaria in urban areas. There are 132 towns in India under the urban malaria control scheme where *A. stephensi* is the vector. *A. stephensi* var. *mysorensis*, a rural inhabitant, and described as a geographical race by Sweet and Rao (1937), has been found to be a vector in Deccan Plateau and Iran (Rao, 1984). Though there was no *A. stephensi* found positive for sporozoites in Mandora, it has been incriminated from several areas in and around Delhi (MRC Annual Report, 1983). In the areas where it has been incriminated, populations were found to be var. *mysorensis* and intermediate type.

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REFERENCES

1. Brown, A.W.A. and R. Pal (1971). Insecticide resistance in Arthropods. (WHO, Geneva).
2. Choudhury, D.S. and S.K. Ghosh (1982). *Plasmodium falciparum* malaria in Haryana villages and a case report of aphasia. *Indian J. Malariol.*, **19**: 69-70.
3. Hemingway, J. (1983). The genetics of malathion resistance in *A. stephensi* from Pakistan. *Trans. R. Soc. Trop. Med. Hyg.*, **77**: 106-108.
4. Manouchehri, A.V., A. Zaini and H. Yazdanpanat (1975). Selection for resistance to malathion in *A. stephensi*. *Mosq. News*, 35: 278-280.
5. Manouchehri, A.V., N. Eshghy and F. Ranhani (1974). Malathion susceptibility test of *Anopheles stephensi mysorensis* in Southern Iran. *Mosq. News*, **34**: 440-442.
6. Rajagopal, R. (1977). Malathion resistance in *A. culicifacies* in Gujarat. *Indian J. Med. Res.*, **66**: 27-28.
7. Rao, T. Ramachandra (1984). ***The Anophelines of India.*** (Rev. ed.).Malaria Research Centre (ICMR), Delhi.
8. Rathor, H.R. and G. Toqir (1980). Malathion resistance in *Anopheles stephensi*. *Mosq. News*, **40**: 526-531.
9. Rathor, H.R. and G. Toqir (1981). Mode of inheritance in *Anopheles stephensi*. *Mosq. News*, **41**: 359-367.
10. Sharma, V.P., D.S. Choudhury, M.A. Ansari, M.S. Malhotra, P.K.B. Menon, R.K. Razdan and C.P. Batra (1983). Studies on the true incidence of malaria in Kharkhoda (District Sonepat, Haryana) and Kichha (District Nainital, U.P.) Primary Health Centres. *Indian J. Malariol.*, **20**(1): 21-34.
11. Subbarao, Sarala K. (1984). Biological species in Malaria Vectors of India. *Proceedings of the Indo-UK Workshop on Malaria, New Delhi, Nov. 14-19, 1983.* Ed. V.P. Sharma. Malaria Research Centre (ICMR), Delhi : 77-83.
12. Subbarao, Sarala K., K. Vasantha, T. Adak and V.P. Sharma (1983). *Anopheles culicifacies* complex. Evidence for a new species, C. *Ann. Entomol. Soc. Amer.*, **76**: 985-988.
13. Subbarao, Sarala K., T. Adak and V.P. Sharma (1980). *Anopheles culicifacies* sibling species

distribution and vector incrimination studies. *J. Com. Dis.*, **12**: 102–104.

14. Sweet, W.C. and B.A. Rao (1937). Races of *A. stephensi* type and var. *mysorensis*. *Indian Med. Gaz.*, **72**: 665–674.

15. WHO/VBC/75.583. Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides.

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Laboratory Experiments on the Effectiveness of Expanded Polystyrene (EPS) Beads in Mosquito Control

V.P. SHARMA[1]

Laboratory tests were done on the usefulness of expanded polystyrene (EPS) beads in mosquito control. It was revealed that the application of EPS beads @ 1gm/240 cm^2 to form 3 to 4 layers on water produced high larval and pupal mortality and prevented mosquitoes from laying eggs. The order of effectiveness was *Culex quinquefasciatus* > *Anopheles culicifacies* > *Anopheles stephensi* > *Aedes aegypti*. The method may be a viable alternative to the current methods used in the control of mosquito breeding, atleast in certain situations.

INTRODUCTION

Mosquito control has become problematical because of the development of insecticide resistance, high cost of insecticides and their application, and the possibility of environmental contamination. In urban areas where vector control measures are directed mainly at the control of immature stages, anti-larval measures are not fully effective, because of the operational problems in the location of mosquito breeding sites and the application of larvicides on weekly basis. Reiter (1978) suggested the idea of applying expanded polystyrene (EPS) beads to form a floating blanket on water as a means to control mosquito breeding and to prevent oviposition, and he reported laboratory tests with *Culex quinquefasciatus*. Expanded polystyrene beads are widely available because of their use in making packaging material and insulation. These are sold at about Rs. 60,000 per metric tonne. A few successful trials on the control of *Culex* breeding using the EPS beads have been completed, and a few more are contemplated mainly in the soakage pits in Tanzania and Kenya (C.F. Curtis, personal communication). The method is simple, inexpensive, safe and does not incur the problem of insecticide resistance. Because of these advantages, further laboratory tests were carried out on the effectiveness of EPS beads in vector control operations. Results of this study are reported in this paper.

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[1]Malaria Research Centre (ICMR)
22, Sham Nath Marg, Delhi-110054, India.

MATERIAL AND METHODS

Laboratory colonized strains of *Anopheles stephensi*, *Anopheles culicifacies*, *Culex quinquefasciatus* and *Aedes aegypti* were used in these studies. Mosquito rearing was carried out following the routine procedure of the

maintenance of colonies as evolved at the Malaria Research Centre. Mosquito larvae, pupae and adults were collected from the insectary and used in these experiments. All tests were carried out at 27±1°C and 70–80% RH. Expanded polystyrene beads, sold by BASF under the trade name of Styropor, were obtained from London. Experiments on the control of mosquito breeding were carried out in 500 ml beakers covered with netting. EPS beads in the size range of 2–4 mm diameter were applied on the water surface @ 1gm/beaker or 240 cm^2 so as to form 3 to 4 layers.

To test the effectiveness of EPS beads in the control of mosquito breeding, 100 mosquito larvae of each instar and 100 pupae were placed separately in beakers in 250 ml water and adequate quantity of larval food added. The water surface in the beakers, with larval instars and pupae, was covered with EPS beads. Concurrently, controls were held without EPS beads. Each experiment with larvae and pupae was replicated 5 times. A record of the mortality of larvae and pupae and of adult emergence was maintained on daily basis and mean values obtained. Oviposition preference of the females, blood fed 2 days before, was studied by placing ovitraps (with or without EPS beads) in the laboratory cages which were being used for the routine collection of eggs, in the insectaries. Estimates of the total number of females in a cage and the number of eggs laid in the ovitraps were made. In another experiment, the capacity of females (blood fed 2 days before) to lay eggs on water surfaces covered with the beads was evaluated. Twenty five females (replicated 5 times) were held in 30×30×30 cm size cages. Each cage was provided with an ovitrap containing beads. An equal number of controls were held without the beads. Eggs were collected daily for 8 days and counted.

RESULTS AND DISCUSSION

Results of the larval and pupal survival are given in Table 1. There was 100% mortality in *Anopheles stephensi*, *Anopheles culicifacies*, *Culex quinquefasciatus* and 98% in *Aedes aegypti* larvae. The 1st and 2nd instars died as they moulted to the next instar. There was no mortality in the controls held without the beads. Tests at the pupal stage revealed very high mortality in pupae of *An. stephensi* (98%), *An. culicifacies* (96.6%) and *Cx. quinquefasciatus* (97%) but relatively low mortality in *Ae. aegypti* (55.2%) pupae. The pupae that survived emerged as normal adult mosquitoes. The mortality in the controls was negligible and varied from 0 to 1.4%.

Results providing a choice for oviposition in untreated ovitraps with a layer of beads are given in Table 2. It was shown that *An. stephensi*, *An. culicifacies* and *Cx. quinquefasciatus* did not oviposit at all on the EPS beads, but some *Ae. aegypti* did so.

Results of oviposition when there was no choice between EPS bead treated and untreated ovitraps are given in Table 3. It was found that while the mosquitoes readily laid eggs on the untreated water surface in the first 2 days after presenting the ovitraps, the number of eggs laid by *An. stephensi*, *An. culicifacies* and *Ae. aegypti* was reduced by 85–95% in the presence of EPS beads and *Cx. quinquefasciatus* did not lay at all. It may be pointed out that the few eggs that were laid by the anophelines and *Ae. aegypti* were laid on the wet beads lying on the edges of the beaker.

The above experiments show that the application of EPS beads was effective in killing immatures and preventing oviposition. This method is environmentally safe and could be applied in the control of mosquito breeding in potable water, as against the present strategy of the treatment of potable water with temephos (Abate), which may be objectionable, and hazardous if carried out by the unskilled worker. It may be pointed out that the method may not be applicable for wells where water is drawn by

Table 1. Mortality of Immatures of Mosquitoes before Adult Emergence after the Application of Expanded Polystyrene Beads

Mosquito species	Mortality (%) of mosquitoes*			
	Larval Instars**		Pupae	
	without beads	with beads	without beads	with beads
An. stephensi	0	100	0.6	98.0
An. culicifacies	0	100	1.0	96.6
Cx. quinquefasciatus	0	100	1.4	97.0
Ae. aegypti	0	98	0	55.2

*Mean of 5 replicates. One hundred each I, II, III & IV instar larvae and pupae were held separately in beakers.
**First and second instar larvae survived initially but died after moulting into next instar. Results of I, II, III & IV instar larvae pooled.

Table 2. Results of Oviposition when Mosquitoes were given a Choice to Oviposit in Ovitraps with or without the Application of EPS Beads

Mosquito species	Approximate number of females held for oviposition	Total eggs collected inside the cages in ovitraps	
		without beads	with beads
An. stephensi	975	15500	0
An. culicifacies	1550	13250	0
Cx. quinquefasciatus	415	191*	0
Ae. aegypti	460	10400	242

*Egg rafts

Table 3. Results of Forced Oviposition when Mosquitoes were held for 8 days to lay Eggs in Ovitraps

Mosquito species (5×25 females of each species)	Number of eggs collected in ovitraps		Reduction (%)
	without beads*	with beads	
An. stephensi	5294	421	92.1
An. culicifacies	3405	178	94.8
Cx. quinquefasciatus	93**	0	100.0
Ae. aegypti	13693	2016	85.3

*All eggs laid in the first 2 days of the experiment.
**Egg rafts

buckets, but may be suitable for disused wells. In water tanks where there is an outflow pipe at the bottom, this would have to be screened to avoid EPS beads going down, if the tank gets emptied. EPS beads have the additional advantage that they are not biodegradable and last for a long time (>2 years, C.F. Curtis personal communication). Since mosquitoes prefer not to lay eggs on the surface of EPS beads, it would be possible to place ovitraps in a nearby site pre-treated with a suitable insecticide. Mosquitoes deterred from laying in a major source by EPS beads would lay eggs in these ovitraps instead of searching for new ovipositional sites, which may be difficult to locate by the anti-larval squads. Larvae in these ovitraps would die on emergence and this would help in reducing mosquito populations.

Use of EPS beads in disease vector control may have its main application in urban areas. Malaria is a serious problem in most cities of India, and for its control the Urban Malaria Scheme (UMS) was launched in 1971–72. At present there are 125 towns containing a total population of 34 million under the UMS (Pattanayak *et al.*, 1981). Similarly filariasis is a serious problem in urban areas. Of the 304 million population living in filaria endemic areas, about 82 million people live in the cities of India. In 1981 there were about 22 million microfilaria positive cases, and 16 million people suffered from the disease in India (Sharma *et al.*, 1983). Epidemics of dengue and its complications occur in urban areas, and the most recent one was in Delhi in 1982 (Uprety *et al.*, 1983). Besides, mosquitoes are so abundant in most towns that their bites and nuisance make life most unpleasant. The common mosquito breeding sites are: overhead tanks, wells, cisterns, roof gutters, barrels, tyre dumps, buckets, tanks, ponds, drains, ditches, borrowpits, rainwater pools, discarded tins etc. At present various methods are used in mosquito control in urban areas. These are cleaning, channelling, filling, larviciding, biological control using *Gambusia* and *Poecilia* fishes etc. Malathion fogging and pyrethrum space spray are also carried out in certain areas but in general larviciding is the main method used in the control of mosquito breeding in urban areas. Mosquito larvicidal oil (MLO), Paris green (copper aceto—arsenate), temephos (Abate) and fenthion (Batyx) are used in the programme (UMS). Larvicidal operations are carried out at weekly intervals throughout the year. These anti-larval measures are not fully effective, as a result mosquito densities are high and mosquito borne diseases are common in urban areas. EPS beads could be used in most of those mosquito breeding sites where the water seldom or never flows or floods. The method would be safe, inexpensive (cost of beads approx. Rs. 5/m^2) and one application alone may last for a long time, thus obviating the need of weekly supervision and treatment. Effectiveness of the EPS beads in the control of mosquito breeding in overhead tanks, cisterns, disused wells etc. is being evaluated in field areas near Delhi. Field trials on the effectiveness of EPS beads in urban malaria control are contemplated in Delhi, Madras, Nadiad and Chandigarh.

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REFERENCES

1. Pattanayak, S., R.G. Roy, K.G. Samnotra and M.S. Bendley (1981). Urban Malaria Scheme of the National Malaria Eradication Programme of India. *Indian J. Malariol.*, **18**(1): 21–27.
2. Reiter, P. (1978). Expanded polystyrene balls: an idea for mosquito control. *Ann. Trop. Med. Parasitol.*, **92**: 595–596.
3. Sharma, S.P., H. Biswas, M. Das and S.R. Dwivedi (1983). Present status of the filariasis problem in India. *J. Com. Dis.*, **15**(1): 53–60.
4. Uprety, H.C., P.K. Srivastava, B.N. Nagpal and V.P. Sharma (1983). Mosquito breeding survey in urban Delhi. *Indian J. Malariol.*, **20**(1): 79–82.

SHORT NOTES

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Outbreak of Malaria in Villages of Bareilly, District U.P.

M.A. ANSARI[1], C.P. BATRA[1] and V.P. SHARMA[1]

Following the reports of deaths due to malaria in Shahjahanpur district villages, it was decided to study the prevalence of malaria in Shahjahanpur affected villages, and in the villages of Bareilly district adjoining the districts of Badaun, Rampur and Pilibhit. Results of the survey of Bareilly district villages are reported in this paper.

Investigations in Shahjahanpur revealed an outbreak of falciparum malaria (Chandrahas and Sharma, 1983). Similar high incidence of malaria was reported from Nainital district (Terai region) which is an adjoining district (Sharma *et al.*, 1983, 1984; Choudhury *et al.*, 1983). Before the survey work started, data on malaria incidence was collected from the District Malaria Officer (DMO). This data showed that the incidence of malaria was almost negligible in Bareilly district i.e., the annual parasite incidence (API) was consistently below 2 ranging from 0.44 to 1.21 during the last five years (1978-83). In order to study the prevailing malaria situation in the neighbouring areas, MRC team randomly selected 12 villages and an urban locality (outskirts of Bareilly), and collected epidemiological and entomological data from 14 to 22 October, 1983.

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[1]Malaria Research Centre (ICMR)
22, Sham Nath Marg, Delhi-110054, India.

Results of parasitological surveys are given in Table 1. A total of 597 blood smears were collected from fever cases. There were 426 malaria parasite positive slides i.e., the slide positivity rate (SPR) was 71.3%. Among the positives 332 *P. falciparum*, 85 *P. vivax* and 9 were mixed infections. The parasite formula was *P. falciparum* 78.4 and *P. vivax* 21.6. Proportion of falciparum malaria was extremely high in all the villages including the urban locality surveyed. Further proof of high endemicity of malaria was obtained by spleen examination of children of the 2 to 12 years age group. The child enlarged spleen rate was 60% in the 96 children examined and the average enlarged spleen was 2.3. During the survey a few cases of deaths, suspected due to malaria were also reported. In the two deaths investigated, the family members of the deceased also had falciparum infection. It was, therefore, likely that the cause of some of the deaths in affected villages was falciparum malaria.

Larval surveys revealed heavy breeding of anophelines in riverbed pools, pits, ponds, seepage water and other water patches. These places were abundant in and around the villages surveyed. Adult collections were carried out in 11 villages. A total of 514 anophelines were collected in 18.30 man hours which comprised of 273 (53.1%) *A. culicifacies*, 153 (29.8%) *A. sub-*

Table 1. Prevalence of Malaria in Villages of Bareilly District

S. No.	Villages	*Malaria cases recorded by (ACD & PCD) NMEP in October, 1983		Blood smears collected	Malaria cases recorded by MRC in a day in each village in October, 1983				Slide Positivity rate	*P. falciparum* relative prevalence (%)
		Pv	Pf		Pv	Pf	Mixed	Total		
	PHC, Fathehganj									
1	Rukumpur	4	4	39	2	27	0	29	74.3	93.1
2.	Madhopur	1	3	28	1	22	1	24	85.7	91.6
3.	Persa Khera	0	0	32	5	22	2	29	90.6	75.8
	PHC, Kyara									
4.	Pepal Gautia	4	1	17	3	1	0	4	23.5	25.0
5.	Kiara	25	3	36	6	7	1	14	38.8	50.0
6.	Manjha	5	1	21	3	5	0	8	38.0	62.5
	PHC, Shergarh									
7	Girdharpur	13	17	48	0	41	1	42	87.5	97.6
	PHC, Majhgawan									
8	Lohari	26	0	135	10	114	3	127	94.07	89.7
	PHC, Bhamora									
9.	Pakhuni	2	1	61	9	45	1	55	90.16	81.8
	PHC, Kuandandha									
10.	Rajpuri	0	0	17	7	2	0	9	52.9	22.2
11.	Palau	0	0	33	12	4	0	16	48.4	25.0
	PHC, Faridpur									
12.	Gojan Sarai	0	0	86	10	24	0	34	39.5	70.5
13.	C.B. Ganj (Bareilly city out-skirt area)	17	0	44	17	18	0	35	79.54	51.4
	Total	97	30	597	85	332	9	426	71.3	77.9

*Malaria cases recorded by the NMEP (ACD and PCD) in the village were 33 in 1981 and 87 in 1982 and no *P. falciparum* case was recorded during this period.

Pv = *P. vivax*

Pf = *P. falciparum*

pictus, 80 (15.6%) *A. annularis*, 5 (1%) *A. fluviatilis*, 2 (0.4%) *A. nigerrimus* and 1 (0.2%) *A. pallidus*. Per man hour density of *A. culicifacies* was 14.9. Of the 243 *A. culicifacies* dissected, two had sporozoites in their salivary glands. *A. culicifacies* was, therefore, responsible for post monsoon transmission of malaria in the villages surveyed. It may be noted that *A. culicifacies* has been extensively incriminated from several areas in north India including the bordering District of Nainital (Choudhury *et al.*, 1983).

Study revealed that the surveillance was totally inadequate and there was an extremely high incidence of malaria, and that DDT spraying was ineffective in controlling mosquito populations. To control malaria, surveillance should be tightened and villages should be sprayed with an effective residual insecticide.

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REFERENCES

1. Chandrahas, R.K. and V.P. Sharma (1984). Malaria epidemic in Shahjahanpur (U.P.). *Indian J. Malariol.*, **20** (2): 163-166.
2. Choudhury, D.S., M.S. Malhotra, R.P. Shukla, S.K. Ghosh and V.P. Sharma (1983). Resurgence of malaria in Gadarpur PHC, district Nainital, Uttar Pradesh. *Indian J. Malariol.*, **20** (1): 49-58.
3. Sharma, V.P., D.S. Choudhury, M.A. Ansari, M.S. Malhotra, P.K.B. Menon, R.K. Razdan, C.P. Batra (1983). Studies on the true incidence of malaria in Kharkhoda (Distt. Sonepat, Haryana) and Kichha (Distt. Nainital, U.P.) Primary Health Centres. *Indian J. Malariol.*, **20** (1): 21-34.
4. Sharma, V.P., M.S. Malhotra and T.R. Mani (1984). Malaria: Entomological and Epidemiological studies in Terai, District Nainital, U.P.: In *Facets of Environmental problems*. Ed. C.R. Krishna Murti. (INSA Publication): 35-45.

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Mosquitoes of Mizoram

P.R. MALHOTRA[1], M. BHUYAN[1] and I. BARUAH[1]

Mosquito survey carried out during April-May, 1979 in Aizawl district of Mizoram, revealed the presence of 26 species belonging to 6 genera, viz., *Anopheles*, *Aedes*, *Armigeres*, *Culex*, *Malaya* and *Mansonia* (Malhotra *et al.*, 1982). As this survey was restricted only to Aizawl distt., due to sensitivity of the area, it was felt necessary to undertake survey in certain other places also, so as to update the information on the mosquito fauna of this union territory.

The present survey was carried out at Zemabawk (Altitude-1074 m), Tuirial (700), Thenzawl (600), Hnathial (824) and Lunglei (1024) during August-September, 1983. Results of these studies are briefly summarised in this paper.

Indoor resting mosquitoes were collected from human dwellings and cattlesheds. Outdoor resting adult mosquitoes were aspirated from open sheds, bushes and vegetation during day time. Mosquitoes were collected by suction tube method. Man biting collections were made by using a good number of baits in unit lines. Battery operated CDC light traps were used from dusk to dawn (5 p.m. to 5 a.m.) in human dwellings, cattle sheds, pigpens, goat-cabins and stables. Larvae were collected from cement tanks, ponds, ditches, river-beds, roadside drains, tree crevices, plant axils, cut bamboos, tyre dumps, earthen pots, used tin containers etc. The larvae were reared in the laboratory set up there until adult emerges. All newly emerged mosquitoes were killed with ether. Common mosquito species were indentified in the field, while other mosquitoes were packed and brought to Defence Research Laboratory, Tezpur (Assam) for their species confirmation and preservation. Mosquitoes were identified with the keys of Christophers (1933), Barraud (1934), Puri (1955), Wattal and Kalra (1967) and catalogue of Knight and Stone (1977).

A total of 1215 mosquitoes were collected and identified into 33 species of 5 genera, viz., *Anopheles*, *Aedes*, *Armigeres*, *Culex* and *Mansonia*. The genus *Anopheles* comprised of 15 species (489 specimens), genus *Aedes* -7 species (97 specimens), genus *Armigeres* - 1 specie (192 specimens), genus *Culex* - 9 species (436 specimens) and genus *Mansonia* - 1 specie (1 specimen). Species composition of the mosquitoes collected is shown in Table 1.

In the fifteen anopheline species collected from Mizoram, the most prevalent species was *A. vagus* (67.07%) followed by *A. maculatus* (12.26%) and *A. aconitus* (7.36%). *A. minimus* was not found in this survey. Among *Culicines*, the most dominant genus was *Culex* and the most prevalent species was *C. tritaeniorhynchus* (27.29%) followed by *C. quinquefasciatus* (26.14%), *C. malayi* (22.24%) and *C. vishnui* (19.03%). Of the *Aedine* mosquitoes, *Aedes*

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[1]Defence Research Laboratory
Post Bag No. 2
Tezpur-784001, India.

Table 1. Results of mosquito collections in Mizoram

Sl No.	Species collected	Total mosquitoes collected
1.	*Anopheles aconitus* Donitz, 1902	36
2.	*A. annularis* Van der Wulp, 1884	2
3.	*A. balabacensis* Baisas, 1936	6
4.	*A. barbirostris* Van der Wulp, 1884	2
5.	*A. culicifacies* Giles, 1901	10
6.	*A. gigas* Giles, 1901	2
7.	*A. jeyporiensis* James, 1902	6
8.	*A. karwari* James, 1903	2
9.	*A. kochi* Donitz, 1901	7
10.	*A. maculatus* Theobald, 1901	60
11.	*A. maculatus* var. *willmorei* James, 1903	13
12.	*A. nigerrimus* Giles, 1900	4
13.	*A. philippinensis* Ludlow, 1902	2
14.	*A. subpictus* Grassi, 1899	9
15.	*A. vagus* Donitz, 1902	328
16.	*Aedes albolateralis* Theobald, 1908	5
17.	*Ae. albopictus* Skuse, 1894 (1895)	24
18.	*Ae. annandalei* Theobald, 1910	16
19.	*Ae. chrysolineatus* Theobald, 1907	40
20.	*Ae. lineatopennis* Ludlow, 1905	1
21.	*Ae. poecilus* Theobald, 1903	6
22.	*Ae. pseudotaeniatus* Giles, 1901	5
23.	*Armigeres subalbatus* Coquillett, 1898	192
24.	*Culex bitaeniorhynchus* Giles, 1901	10
25.	*C. fuscanus lutzia* Wiedmann, 1820	1
26.	*C. gelidus* Theobald, 1901	9
27.	*C. malayi* Leicester, 1908	97
28.	*C. mimulus* Edwards, 1915	2
29.	*C. quinquefasciatus* Say, 1823	114
30.	*C. sinensis* Theobald, 1903	1
31.	*C. tritaeniorhynchus* Giles, 1901	119
32.	*C. vishnui* Theobald, 1901	83
33.	*Mansonia uniformis* Theobald, 1901	1
	Total	1215

chrysolineatus formed 42.23% of the total. *Ae. albopictus* was the next dominant species. The population of *Armigeres subalbatus* was fairly good.

Presence of *A. dthali* Patton, 1905 and *A. jamesi* Theobald, 1901 was reported by Pandiya (personal communication) during his studies in June, 83. Recently, Das and Barua reported the existence of *A. majidi* Mc Combie Young and Majid, 1928 and *A. minimus* Theobald, 1901 in Tuichang, a border town in south Mizoram, during malaria transmission studies in the area in July, 84 (unpublished report). Malhotra *et al.* (1982) reported the presence of *A. hyrcanus* in the study area. Rao (1984) is of the view that no "true *A. hyrcanus*" exist in India. In fact, "*A. hyrcanus*" is a group consisting of more than ten species, of which *A. argyropus*, *A. nigerrimus*, *A. nitidus*, *A. sinersis*, *A. peditaeniatus* and *A. crawfordi* are recorded in India. According to his key, the species reported earlier as *A. hyrcanus* by Malhotra *et al.* (*loc. cit.*) is actually *A. nigerrimus*.

Thus mosquito fauna of Mizoram swells up to 45 species of 6 genera, viz., *Anopheles* (19 species), *Aedes* (8 species), *Armigeres* (1 specie), *Culex* (14 species), *Malaya* (1 specie) and *Mansonia* (2 species).

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REFERENCES

1. Barraud, P.J. (1934). *The fauna of British India including Ceylon and Burma, v. 5.* (Taylor and Francis, London): 1-463.
2. Christophers, S.R. (1933). *The fauna of British India including Ceylon and Burma, v. 4.* (Taylor and Francis, London): 1-360.
3. Knight, K.L. and A. Stone (1977). *A catalog of the mosquitoes of the world (Diptera : Culicidae).* (The Geo. W. King Company, Baltimore, Maryland 21202): 1-558.
4. Malhotra, P.R., B.C. Chakraborty, N.G. Das and P.K. Sarkar (1982). Mosquito survey in Aizawl district (Mizoram). *J. Assam Sci. Soc.*, 25(1): 82-85.
5. Puri, I.M. (1955). Synoptic table for the identification of the Anopheline Mosquitoes of India. *Health Bulletin No. 10.*
6. Rao, T.Ramachandra (1984). *The Anophelines of India.* (Rev. ed.). Malaria Research Centre (ICMR), Delhi.
7. Wattal, B.L. and N.L. Kalra (1967). Regionwise pictorial keys to the female Indian *Anopheles.* (Indian Society for Malaria and other Communicable Diseases, Delhi): 46-54.

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Evaluation of Micro-sampling of Blood by Filter Paper Strips for Malaria Seroepidemiology

H. SRINIVASA[1] and P. BHAT[1]

In India, sero-epidemiology of malaria is gaining recognition as an important research tool with the onset of resurgent malaria. This requires application of serological tests on a large number of serum samples collected during the surveys. The method of micro-sampling of blood has made this possible. Micro-sampling can be done either using preheparinised capillary tubes (WHO, 1974; Gupta *et al.*, 1983) or by using strips of filter paper (Collins *et al.*, 1971; Kagan, 1972; Draper, 1979; Lobel *et al.*, 1976). By the former method neat plasma can be obtained and there is scope for repetition of a serological test. The latter method yields diluted blood, but is more widely followed as it is simple to collect and transport blood samples (Kagan, 1972). In the present study a comparative evaluation of both these micro-sampling techniques was carried out using Indirect fluorescent antibody test (IFA) in Hosadurga, a town in Chitradurga district of Karnataka State, southern India. It is an area endemic for malaria (Annual Parasitic Index for 1982 = 20/1000; *Plasmodium vivax* = 96.8%, *P. falciparum* = 3.2%: Data obtained from District Hlth & F.W. Officer, Chitradurga District, Karnataka). A total of 94 persons drawn from different age groups and both sexes were selected. From each person blood was collected by finger prick method sequentially in a pre-heparinised capillary tube and on a strip of filter paper.

Collection of blood in pre-heparinised capillary tubes: Locally available conical tipped capillary tubes having a length of 80 mm and an internal diameter of 2 mm (specifications as per Dr. Lobel, CDC, Atlanta, USA - personal communication) were heparinised by rinsing once with heparin solution (1000 IU/ml). Blood was collected by finger prick technique in these pre heparinised capillary tubes. The labelled capillary tubes were transported to the laboratory situated in Bangalore where plasma samples were separated without further delay into labelled vials containing 10 µl of 1% sodium azide. These samples were designated as capillary plasma and stored at -20°C. Volume of plasma collected varied between 200-300 µl.

Collection of blood on filter paper strips: Blood collection and subsequent elution from filter papers were done as per the protocol of CDRI, Lucknow (G.P. Dutta, personal communication). In brief, strips of Whatman 3 mm chromatography paper having a length of 5 cm and a breadth of 3 cm were used. The blood obtained

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[1]Department of Microbiology
St. John's Medical College
Bangalore-560034, India.

by finger prick was absorbed on this strip so as to obtain a thoroughly soaked spot with a diameter of 10 mm. It is found that this corresponds to 50 µl of blood of 25 µl of serum considering 50% haematocrit. The blood spots were dried at ambient temperature, the strips were coded, sealed in plastic bags and these were transported to the laboratory where they were stored at 20°C.

For elution, each blood spot was cut out of the filter paper strip and the disc obtained was immersed in a test tube containing 200 µl of PBS-pH 7.2. The test tubes were kept for 60 mins. at room temperature. The filter paper eluate constitutes 1:8 serum dilution which was adjusted to 1:10 and two fold dilutions of this were used for the test.

Control filter paper strip: In order to check on the efficiency of filter paper method, 50 µl of a serum having a IFA titre of 1:1280 was spotted on a filter paper strip over an area of 10 mm. The elution was done as described above.

IFA for the detection of malaria antibodies: IFA was done as described earlier (Gupta *et al.*, 1981) using *in vitro* cultured *P. falciparum* as the antigen. A titre of ≥1:40 was considered as positive. In addition to 94 capillary plasma and corresponding filter paper eluates, the eluate from the control filter paper strip was also included in the test.

Results are shown in Table 1. While 18/94 Capillary plasma were positive, only 8/94 filter paper eluates were positive. The difference was statistically significant (z=5.05; significant at 1% level). Comparison of the reciprocal antibody titres revealed that maximal antibody titre with capillary sample was 1280 while with filter paper eluates it was only 40 (Table 1.). In addition there were as many as 12 capillary samples with a titre of 80. The eluate from the control filter paper gave a reciprocal antibody titre of 160 in IFA.

Thus in the present study filter paper method was less sensitive for assay of malarial antibodies. Similar results have been noted earlier by Kagan, 1972 and Lobel *et al.*, 1976 who have used filter eluates for IHA test. The fact that control filter paper strip gave a decreased IFA titre in this report further indicates the reduced sensitivity of the method. It is also reported that absorption of blood on the filter paper may destroy the reactivity of IgM class of antibodies (WHO, 1974). In summary, it can be said that for serology of malaria particularly when it is used to evaluate antimalarial measures in areas of disappearing malaria, a sensitive method of microblood sampling like the capillary method is essential.

Table 1. Comparative antibody titres of capillary plasma and filter paper eluates of Hosadurga 1982-83 by IFA using *in vitro* cultured *P. falciparum*

	Reciprocal antibody titres	Capillary plasma								
		< 20	20	40	80	160	320	640	1280	Total
Filter paper eluates	<20	56	5	4	3	2	1	-	1	72
	20	8	1	-	3	-	2		-	14
	40	2	4	2	-	-	-	-	-	8
	80	-	-	-	-	-	-	-	-	-
	160	-	-	-	-	-	-	-	-	-
	320	-	-	-	-	-	-		-	-
	640	-		-	-	-	-	-	-	-
	1280	-			-	-	-		-	-
Total		66	10	6	6	2	3	-	1	94

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REFERENCES

1. Collins, W.E., M. Warren and J.C. Skinner (1971). Serological malaria survey in the Ethiopian Highlands. *Am. J. Trop. Med. Hyg.*, **20**: 199–205.
2. Draper,C.C. (1979). In *Immunodiagnostic techniques in Malaria* – Transactions of the third meeting of the Scientific Working Group on the Immunology of Malaria held under the auspices of UNDP/World Bank/WHO (Panama): 155–162.
3. Gupta, M.M., H. Srinivasa and P. Bhat (1983). Seroepidemiology of Malaria in Karnataka State: Longitudinal study of a population from an area with high incidence at Kolar, South India. *J. Inf. Dis.* **148**: 609.
4. Gupta, M.M., M.J. Sebastian, P. Bhat and H.O. Lobel (1981). Evaluation of *in vitro* cultured *Plasmodium falciparum* as antigen for malaria serology. *J. Trop. Med. Hyg.*, **84**: 165–170.
5. Kagan, I.G. (1972). Evalutaion of indirect haemagglutination test as an epidemiologic technique for malaria. *Am. J. Trop. Med. Hyg.*, **21**: 683–698.
6. Lobel, H.O., A.J. Najera, Wan I. Ch'en, P. Munroe and H.M. Mathews (1976). Seroepidemiologic investigations of malaria in Guyana. *J. Trop. Med. Hyg.*, **79**: 276–284.
7. WHO Memorandum (1974). Serological testing in malaria. *Bull. WHO.* **50**: 527–535.

OBITUARIES

Smt. Indira Gandhi

Architect of Indian Science

(1917–1984)

Science and technology in India has been dealt the severest blow by the sudden death of Prime Minister Indira Gandhi. The phenomenal growth of scientific research and development in independent India is closely identified with the active support of the first Prime Minister of independent India, Jawaharlal Nehru and later by his illustrious daughter Smt. Indira Gandhi. They sincerely believed that the only way to get India out of the shackles of poverty is by pursuing a policy of self-reliance in which science and technology have a vital role to play. Both of them visualized science as a powerful instrument of social change and were deeply committed to this cause.

Smt. Gandhi's concern for the health and well-being of the people of India is well known. She was deeply concerned with the under-privileged and her New 20-Point Programme emphasized universal primary health care facilities with particular reference to the welfare of children, pregnant women and nursing mothers. We at the ICMR fondly remember her visit to the Headquarters on 22nd April, 1983 when she showed great interest and enthusiasm regarding the recent advances in medical research and urged the scientists to actively assist and steer the national health programmes. She always felt that research and development should go hand in hand.

As the 'architect of modern science' in India, Indira Gandhi's commitment and support to scientific community was legendary. Her enthusiasm and support was fully reciprocated by the scientists of India who have succeeded in placing India on the scientific map of the world and even brought it to the stage of being called the "scientific superpower of the third world". The ICMR scientists rededicate themselves to strive hard to achieve the objective of the overall betterment of human life—a goal which was so close to Smt. Gandhi's heart.

V. Ramalingaswami
Director-General
Indian Council of Medical Research
New Delhi

Paul Farr Russell*

(1894–1983)

Dr. Paul Farr Russell, one of the world's leading malariologist, died in Richmond, Virginia, in his 89th year.

The son of a Baptist minister, Paul Russell was born in Boston, Mass. in 1894 [August 12] and received his medical degree at Cornell University Medical School. Encouraged by his devout parents, the young doctor decided that he would employ his professional knowledge to help the sick people in distant parts of the world. After completing two years of internship at the Bellevue Hospital in New York, Paul joined the Rockefeller Foundation in 1923 as staff member of the International Health Division. His first field assignment was to Singapore and to Malaya, to carry out the Foundation's rural sanitation programme, linked with the attempted eradication of hookworm. He was then transferred to the Philippines to study and control malaria, a serious local problem. From then on Russell committed himself wholeheartedly to research and fight against this disease. In 1935, having spent 5 years in the Philippines, where he carried out a remarkable study of rural malaria, Russell went to India, to set up at the King's Institute in Madras a malaria research station of the Rockefeller Foundation. An important method of malaria control by pyrethrum spraying was developed there by Russell and his Indian colleagues. When World War II broke out Russell joined the U.S. Army Medical Corps and, after having spent a brief period of duty in Panama and Puerto Rico, was transferred, in 1942 at General MacArthur's request to his headquarters in Australia. Colonel Russell played an important role in planning and supervising control operations in the South Pacific theatre of war, where malaria admission rates in the American forces averaged in 1942 some 670 per 1,000. Without the principles developed by Russell the victory in the Pacific would have been much more difficult and more costly in human lives. Within a year the situation improved dramatically and in 1943 Russell was transferred to North Africa as Chief Malariologist Allied Forces HQ and a year later became Chief Malariologist, Allied Control Commission

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of the Italian-Mediterranean Area. For this work Russell was awarded by the President of the U.S.A. the Legion of Merit. After the war Russell became one of the leaders of the international effort for research and control of malaria and was one of the members of the first Expert Committee on Malaria set up in 1946 by the Interim Commission, which prepared the constitution of the World Health Organization. Soon he was the moving spirit of the World Health Organization's plans for global eradication of malaria. It was under Russell's guidance that the WHO Expert Committee prepared in 1956 in Athens, a report that became a bible of malaria eradication for the next decade. In 1953, Russell gave a remarkable series of Heath-Clark lectures at the University of London; this was later published under the title *Man's Mastery of Malaria*, an erudite and evocative history of malariology. In 1949 the American Society of Tropical Medicine awarded him the Walter Reed Medal and in 1957 he received from the World Health Assembly the prestigious Darlin Medal and Prize. His *Practical Malariology* first published in 1946, saw a second edition in 1963 and remains the most complete guide of malaria control. During the next 15 years Russell continued writing, travelling, corresponding and participating in the work of the U.S.A. National Research Council and the World Health Organization, but the lack of complete success of the malaria eradication programme and the recent reverses of malaria control in many parts of the world were to him a source of deep personal disappointment.

After his retirement from the Rockefeller Foundation he became visiting professor at the Harvard School of Public Health. The American Society of Tropical Medicine and Hygiene awarded him in 1979 the Le Prince Medal for his services to malaria control. He received the Mary Kingsley Medal of the Liverpool School of Tropical Medicine and was Honorary Fellow of the Royal Society of Tropical Medicine and Hygiene and of many other societies.

A few years ago he moved from his old home in Maine to the warmer climate of Virginia. His robust health began to deteriorate after a brief but painful illness which affected his eyesight, but he was still keeping up his interest in malaria research, through correspondence with his friends and former students.

He died peacefully on 2nd November, 1983, at St. Luke's Hospital in Richmond, Virginia. Besides his good looks and a segnieurial bearing, Paul Russell had an unforgettable personality. His erudition was prodigious and extended to all aspects of public health and to history of medical sciences. A man of outstanding scientific probity, lively intelligence, remarkable capacity for work and high moral principles, he was kind and helpful to all who worked with him, but could be firm and yet invariably courteous. His friendship once given was enduring and generous. Like Ronald Ross at the beginning of this century, Paul Russell felt that science is worth pursuing only if it will be in the service of man. In practical terms it meant to him, developing methods of malaria control that could be of direct value to rural populations of the Third World.

His death closes one of the visionary and heroic chapters in the history of tropical medicine and public health. His innumerable friends all over the world will remember him with respect and great affection. They share their sorrow with Mrs. Phyllis Russell and with his sons, Christopher and Theodore, both in the foreign service of the U.S.A.

Leonard J. Bruce-Chwatt
Wellcome Museum of Medical Science
183 Euston Road
London NW1 2BP, England

T. Ramachandra Rao

(1907–1984)

In the passing away of Dr. T. Ramachandra Rao on 8th November, 1984 in Bangalore, the country has lost an eminent malariologist.

Dr. T. Ramachandra Rao was born in Mysore on 9th October, 1907. His father Sri R. Thammajee Rao was a member of the Mysore State Civil Service. Dr. Ramachandra Rao received his early education in Mysore and Bangalore, and graduated in Science from the Central College, Bangalore. He got his Master's Degree in Zoology with distinction from the University of Calcutta. Thereafter, he served as Lecturer for two years at the Mysore Medical College. He obtained his doctorate in Science from the University of Calcutta in 1951.

Dr. Ramachandra Rao joined the Rockefeller Foundation in 1936 and worked on the cytogenetics of grasshoppers. He soon shifted to work with Dr. Paul Russell, on the bionomics and control of mosquitoes. Together, they carried out extensive and intensive studies on *Anopheles culicifacies*, a major vector of malaria in India. They also worked extensively on irrigation malaria, and on the development of spray killing technique.

In 1942, Dr. Ramachandra Rao joined the newly created Malaria Organisation of Bombay State as an entomologist and rose to become the Deputy Director of Public Health of the Maharashtra State in-charge of malaria and filariasis. He took part in the largest public health programme undertaken by the Government of India in rural malaria control, and carried out numerous studies on the bionomics and control of *Anopheles fluviatilis*, culminating in the demonstration of the feasibility of rural malaria control with DDT. His work with Dr. D.K. Vishwanathan established the norms later largely adopted by the National Malaria Control Programme and the National Malaria Eradication Programme. His studies on this aspect have been hailed internationally.

Dr. Rao spent a year in 1945 at the London School of Hygiene and Tropical Medicine, working with the late Prof. P.A. Buxton, FRS.

For 9 years, from 1961 to 1970, Dr. Rao was the Director of the Virus Research Centre (now the National Institute of Virology, Pune), then run jointly by the Indian Council of Medical Research and Rockefeller foundation, where he conducted and guided research on the epidemiology of arboviruses and helped in achieving an international status for the Institute. Later he worked as Officer on Special Duty at the ICMR Headquarters for three years. Dr. Ramachandra Rao was consultant and advisor to ICMR for over 35 years in many matters connected with communicable diseases. World Health Organisation also utilized his services as the Team Leader in Afghanistan and Tanzania. He was a member of the WHO expert committees and study groups on malaria, insecticides, vector biology and control, and virus diseases. He had well over 120 original research publications to his credit including the monograph on *The Anophelines of India*. He was a Fellow of the Indian National Science Academy, National Academy of Medical Sciences (India), Indian Society for Malaria and other Communicable Diseases and the Royal Society of Tropical Medicine and Hygiene, London. He was also an honorary member of the American Society of Tropical Medicine and Hygiene.

Dr. Ramachandra Rao was president of the Zoology and Entomology Section of the Indian Science Congress in 1972, and chairman of several international and national scientific meetings. He delivered the Sharada Devi Paul Memorial Oration at NIV Pune, T.S. Narayana Rao Oration Award ICMR (1974) and Sisir Kumar Mitra Lecture INSA (1978). He was also the team leader for the in-depth evaluation of NMEP in 1970.

Dr. Ramachandra Rao was intimately associated with the research and control of malaria until his death. He was a person who commanded uniform respect from his colleagues and assistants, and also from the scientific community. He is survived by his wife, three daughters and two sons.

V.P. Sharma
Malaria Research Centre (ICMR)
22-Sham Nath Marg
Delhi-110054, India.

Books/Monographs

Rao, T.R. (1981). *The Anophelines of India.* (W.Q. Judge Press, Bangalore).

Landau, I. and Y. Boulard (1978). In *Rodent Malaria,* edited by R. Killick-Kendric and W. Peters.(Academic Press Inc., London): 53–84.

Paper presented at Symposium/Conference

Subbarao, S.K. *Cytoplasmic incompatibility in mosquitoes.* Paper presented at the International symposium on recent developments in the genetics of insect disease vectors, Bellagio, Italy, 20–24 April, 1981.

8. Short notes should be prepared in a manner similar to the research papers and will have title, name(s) of author(s) with address of Institute/University as footnotes, acknowledgement and references.

9. Reviews would be published of the invited papers only. Book reviews may be published at the end of the journal.

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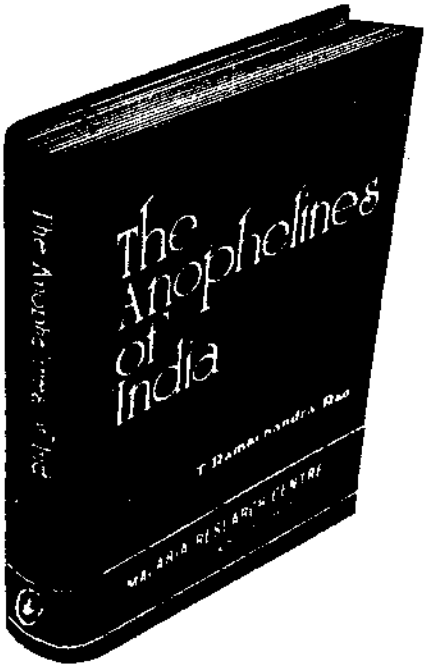

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